

University of Bath



## DOCTOR OF MEDICINE

**Seroprevalences of atypical respiratory infections, SeroCp ELISA reproducibility, electrocardiographic rhythm and ischaemic changes, socioeconomic deprivation and survival outcome in elderly stroke and control medical patients**

Ngeh, Joseph

*Award date:*  
2008

*Awarding institution:*  
University of Bath

[Link to publication](#)

### General rights

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal ?

### Take down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

**Seroprevalences of atypical respiratory infections,  
SeroCp ELISA reproducibility, electrocardiographic  
rhythm and ischaemic changes, socioeconomic  
deprivation and survival outcome in elderly stroke  
and control medical patients**

**Joseph Kho Tong Ngeh, FRCP**

**A thesis submitted for the degree of Doctor of Medicine**

**University of Bath**

**School For Health**

**January 2008**

**COPYRIGHT**

Attention is drawn to the fact that copyright of this thesis rests with its author and publishers of published works.

This copy of the thesis has been supplied on condition that anyone who consults it is understood to recognise that its copyright rests with its author and publishers of published works and that no quotation from the thesis and no information derived from it may be published without the prior written consent of the author and publishers.

This thesis may be made available for consultation within the University Library and may be photocopied or lent to other libraries for the purposes of consultation.



## Abstract

This thesis was based on a collection of my published works. Chapter 1 introduced the idea that infections, both acute and chronic, were risk factors for stroke. Various infections and micro-organisms associated with stroke were discussed. Specifically, the relationship between micro-organisms such as *Cytomegalovirus*, *Helicobacter pylori*, dental pathogens, and stroke were discussed. The theories of the pathogenesis of atherosclerosis were presented. Chapter 1 also reviewed the association between *Chlamydia pneumoniae*, an atypical respiratory pathogen, and atherosclerosis in detail.

Chapter 2 introduced the ‘*Chlamydia pneumoniae* in elderly patients with stroke’ or ‘C-PEPS’ study from which published works in subsequent chapters of this thesis were based. The C-PEPS was a case-control study that investigated the seroprevalence of *C pneumoniae* in 100 elderly acute stroke and transient ischaemic attack (TIA) patients versus 87 control medical patients. The C-PEPS study showed a high seroprevalence (immunoglobulin IgG) of *C pneumoniae* infection in both cases and controls. There was no significant association between *C pneumoniae* seropositivity and stroke / TIA.

Chapter 3 presented a study that investigated the reproducibility of a commercial enzyme linked immunosorbent assay (ELISA) kit (SeroCP, Savyon) used in the C-PEPS study. The study concluded that SeroCP ELISA had a good reproducibility for the detection of *C pneumoniae* IgA and moderately good reproducibility for *C pneumoniae* IgG and IgM.

In chapter 4, the ‘*Mycoplasma pneumoniae* in elderly patients with stroke’ or ‘M-PEPS’ case-control study was based on the same cohort of patients as in the C-PEPS study. The M-PEPS study showed a high seroprevalence of *M pneumoniae*, another atypical respiratory pathogen, in the cohort. However, the study had ruled out *M pneumoniae* as a major risk factor for stroke / TIA.

In chapter 5, the ‘*Legionella pneumophila* in elderly patients with stroke’ or ‘L-PEPS’ was another case-control study based on the same cohort of patients as in the C-PEPS

study. The L-PEPS study established that there was no statistical difference between the seroprevalence of *L pneumophila*, another atypical respiratory pathogen, in both the stroke / TIA patients and control medical patients. However, when the results of C-PEPS, M-PEPS and L-PEPS studies were analysed together, it appeared that the aggregate number or infectious burden of chronic atypical respiratory infections was associated with the risk of stroke / TIA.

Chapter 6 presented a pilot study that investigated the seroprevalence of *Coxiella burnetii*, another atypical respiratory pathogen, in the same cohort of patients as in the C-PEPS study. Due to very low seropositivity and zero counts, a conclusion could not be made on any association between *C burnetii* seropositivity and stroke / TIA.

Chapter 7 presented the electrocardiographic findings such as rhythms and ischaemic changes of the same cohort of patients as in the C-PEPS study. Atrial fibrillation was the commonest rhythm abnormality in both elderly stroke / TIA cases and medical controls. After adjustment for background history of ischaemic heart disease, there was a statistical trend to suggest an association between ischaemic electrocardiographic changes and stroke / TIA.

Chapter 8 presented a case-control study that investigated the relationships between socioeconomic deprivation (SED) status, atypical respiratory infections and survival outcome in the same cohort of patients as in the C-PEPS study. The SED status of stroke / TIA cases and controls were similar. Although an association between acute atypical respiratory infection and SED was only found in the control medical patients, acute atypical respiratory infectious burden was found to associate with income deprivation in the whole cohort of elderly stroke / TIA and medical patients. SED on its own had only a modest effect on the association between chronic atypical respiratory infectious burden and stroke / TIA. Elderly patients' duration of survival after an acute stroke / TIA appeared not to be affected by their background SED status.

Chapter 9 was the concluding chapter. The main findings of the published works submitted for the thesis were summarised and discussed. Some directions for future research were also discussed.

## **Dedication**

**To my wife Vivien, our children and parents**

# Contents

<b>Abstract</b> .....	2
<b>Table of contents</b> .....	5
<b>List of figures</b> .....	9
<b>List of tables</b> .....	10
<b>List of published works submitted for / relevant to the thesis</b> .....	13
<b>Declaration of materials from a previously submitted dissertation</b> .....	15
<b>Declaration of published works done in collaboration with others</b> .....	16
<b>Acknowledgements</b> .....	22
<b>Abbreviations</b> .....	24

## Chapter 1

<b>Infections as risk factor for stroke: focussing on the association between <i>Chlamydia pneumoniae</i> and atherosclerosis</b> .....	28
1.1. Introduction .....	29
1.2. Pathological causes of stroke .....	30
1.3. Acute infection and stroke .....	31
1.3.1. Acute systemic infection and stroke .....	32
1.3.2. Acute central or local infection and stroke .....	34
1.3.3. Infective endocarditis and stroke .....	36
1.4. Mechanisms of infection-associated stroke .....	37
1.5. Pathogenesis of atherosclerosis .....	39
1.5.1. Definition of atherosclerosis .....	39
1.5.2. Theories of atherogenesis .....	39
1.6. Chronic infection and atherosclerosis or stroke .....	43
1.6.1. <i>Cytomegalovirus</i> .....	45
1.6.2. <i>Helicobacter pylori</i> .....	46
1.6.3. Periodontal pathogens .....	48
1.7. <i>Chlamydia pneumoniae</i> .....	50
1.7.1. Introduction .....	50
1.7.2. Microbiology and epidemiology of <i>C. pneumoniae</i> .....	51
1.7.3. Evidence linking <i>C. pneumoniae</i> and atherosclerosis: .....	54

1.7.3.1. Seroepidemiological observations .....	54
1.7.3.2. Pathology specimen findings .....	57
1.7.3.3. Animal experimental models .....	62
1.7.3.4. Molecular and immunological mechanisms .....	65
1.7.3.5. Antibiotic treatment studies .....	72
1.7.4. <i>C. pneumoniae</i> , atherosclerotic risk factors and Koch's postulates .....	86
1.8. Concluding comments .....	89

## Chapter 2

<b>An introduction to the '<i>Chlamydia pneumoniae</i> in elderly patients with stroke' (C-PEPS) study .....</b>	<b>90</b>
2.1. Introduction .....	91
2.2. Subjects and Methods .....	92
2.3. Serological analysis .....	93
2.4. Statistical methods .....	94
2.5. Ethics committee approval .....	95
2.6. Results .....	95
2.7. Discussion .....	102
2.8. Aims of further published works and thesis .....	106

## Chapter 3

<b>The reproducibility of SeroCP enzyme-linked immunosorbent assay (ELISA) for detection of <i>Chlamydia pneumoniae</i>-specific antibodies .....</b>	<b>108</b>
3.1. Introduction .....	109
3.2. Patients and Methods .....	109
3.3. Results .....	111
3.4. Discussion and conclusions .....	116

## Chapter 4

<b><i>Mycoplasma pneumoniae</i> in elderly patients with stroke: a case-control study on the seroprevalence of <i>Mycoplasma pneumoniae</i> in elderly patients with acute stroke / transient ischaemic attack. The M-PEPS study .....</b>	<b>121</b>
4.1. Introduction .....	122
4.2. Subjects and Methods .....	123

4.2.1. Serological analysis .....	124
4.2.2. Statistical methods .....	125
4.2.3. Ethics committee approval .....	125
4.3. Results .....	126
4.4. Discussion .....	130
4.5. Conclusions .....	136

## Chapter 5

### ***Chlamydia pneumoniae*, *Mycoplasma pneumoniae* and *Legionella pneumophila* in elderly patients with stroke (C-PEPS, M-PEPS, L-PEPS): a case-control study on the infectious burden of atypical respiratory pathogens in elderly patients with acute stroke / transient ischaemic attack .....**

5.1. Introduction .....	139
5.2. Subjects and Methods .....	140
5.2.1. Serological analysis .....	141
5.2.2. Statistical methods .....	142
5.3. Results .....	142
5.4. Discussion .....	153
5.5. Conclusions .....	158

## Chapter 6

### ***Coxiella burnetii* in elderly patient with stroke (C-BEPS): a case-control study on the seroprevalence of *Coxiella burnetii* in elderly patients with acute stroke / transient ischaemic attack .....**

6.1. Introduction .....	161
6.2. Subjects and Methods .....	162
6.3. Results .....	162
6.4. Discussion .....	163

## Chapter 7

### **A case-control study on the prevalence of electrocardiographic rhythms and ischaemic changes in elderly patients with acute stroke / transient ischaemic attack .....**

7.1. Introduction .....	169
-------------------------	-----



7.2. Methods .....	169
7.3. Results .....	170
7.4. Discussion .....	172
7.5. Conclusions .....	181

## **Chapter 8**

### **The relationship between socioeconomic deprivation, atypical respiratory infections and survival outcome in elderly stroke and medical patients .....**

8.1. Introduction .....	184
8.2. Subjects and Methods .....	185
8.2.1. Socioeconomic status .....	185
8.2.2. Survival outcome .....	187
8.2.3. Statistical analyses .....	187
8.3. Results .....	188
8.4. Discussion .....	204
8.4.1. Limitations .....	211
8.5. Summary .....	213

## **Chapter 9**

### **General discussion of published works and conclusions .....**

9.1. Introduction .....	216
9.2. Infections and stroke .....	216
9.3. <i>Chlamydia pneumoniae</i> and atherosclerosis .....	217
9.4 The C-PEPS study .....	220
9.5. SeroCP ELISA reproducibility .....	222
9.6. The M-PEPS study .....	223
9.7. The L-PEPS study .....	224
9.8. Atypical respiratory infectious burden and stroke .....	225
9.9. The C-BEPS study .....	226
9.10. ECG and stroke .....	227
9.11. Socioeconomic deprivation, atypical respiratory infections, survival outcome and stroke .....	228
9.12. Conclusions .....	230

## **Appendix A**

Principle and procedure of Enzyme-Linked Immunosorbent Assay (ELISA) test ...233

## **Appendix B**

ELISA test validation, calculation and interpretation of results .....235

*Chlamydia pneumoniae*-SeroCP ELISA (Savyon, Israel) .....235

*Mycoplasma pneumoniae*-SeroMP ELISA (Savyon, Israel) .....236

*Legionella pneumophila*-ELISA (VIRCELL SL, Spain) .....237

*Coxiella burnetii*-ELISA (PANBIO, Australia) .....238

## **Appendix C**

List of relevant oral and poster presentations, and peer-reviewed abstract publications .....239

**Bibliography** .....240

## **List of Figures**

### **Chapter 1**

Figure 1. Life cycle of *C pneumoniae* .....53

Figure 2. Transmission electron micrograph of smooth muscle cells in an early atherosclerotic lesion of the aorta positive for *C pneumoniae* by polymerase chain reaction .....59

Figure 3. The roles of *C pneumoniae*, monocytes, and inflammatory markers in atherogenesis and atherothrombosis: interplay between molecular and immunological mechanisms.....71

### **Chapter 8**

Figure 1a. Kaplan-Meier plot for stroke / TIA cases and medical controls .....196

Figure 1b. Kaplan-Meier plot for stroke / TIA cases (IMD scores in tertiles) .....197

Figure 1c. Kaplan-Meier plot for medical controls (IMD scores in tertiles) .....198

Figure 1d. Kaplan-Meier plot for stroke / TIA cases (IDAOPi scores in tertiles) ....199

Figure 1e. Kaplan-Meier plot for medical controls (IDAOPi scores in tertiles) .....200

Figure 1f. Kaplan-Meier survival curves according to the number of atypical respiratory pathogens for IgG seropositivity in cases and controls .....	202
Figure 1g. Kaplan-Meier survival curves according to the number of atypical respiratory pathogens for IgM seropositivity in cases and controls .....	203

## List of Tables

### Chapter 1

Table 1. Microorganisms implicated to associate infections with atherosclerotic vascular diseases .....	43
Table 2. Pathological studies that have detected or cultured viable <i>C pneumoniae</i> from human atherosclerotic tissues .....	60
Table 3. Antibiotic treatment studies and atherosclerotic vascular diseases .....	72
Table 4. Koch's postulates for infectious diseases: <i>Chlamydia pneumoniae</i> in atherosclerosis versus <i>Helicobacter pylori</i> in peptic ulcer disease .....	87

### Chapter 2

Table 1. Distribution of risk factors in stroke / TIA cases and controls .....	96
Table 2. Seroprevalence of <i>Chlamydia pneumoniae</i> antibody in cases and controls .....	97
Table 3. Odds ratios for stroke / TIA in relation to serological markers of <i>C pneumoniae</i> infection .....	98

### Chapter 3

Table 1a. Cut-off index (COI) of samples with borderline results in the first run and their corresponding COI in the second run .....	112
Table 1b. Cut-Off Index (COI) of samples with borderline results in the second run and their corresponding COI in the first run .....	112
Table 1c. Mean Cut-Off Index (COI) of samples with borderline results in either the first or second runs and mean of their corresponding COI .....	112
Table 2. Cut-Off Index of samples giving discrepant results in the first and second runs .....	113

Table 3. SeroCP ELISA reproducibility .....	114
---	-----

## Chapter 4

Table 1. Distribution of risk factors in 95 stroke / TIA cases and 82 controls .....	126
Table 2. Seroprevalence of <i>M pneumoniae</i> antibody in cases and controls .....	127
Table 3. Odds ratios for stroke / TIA in relation to serological markers of <i>M pneumoniae</i> infection .....	128

## Chapter 5

Table 1. Distribution of risk factors in 91 stroke / TIA cases and 86 controls .....	142
Table 2. Seroprevalence of <i>L pneumophila</i> antibody in cases and controls .....	143
Table 3. Odds ratios for stroke / TIA in relation to serological markers of <i>L pneumophila</i> infection .....	144
Table 4. Odds ratios for stroke / TIA in relation to IgG or IgM seropositivity for 0, 1, 2 or 3 of <i>C pneumoniae</i> , <i>M pneumoniae</i> , and <i>L pneumophila</i> .....	146
Table 5. Odds ratios for stroke / TIA in relation to specific combinations of <i>C pneumoniae</i> - <i>M pneumoniae</i> , <i>M pneumoniae</i> - <i>L pneumophila</i> , and <i>L pneumophila</i> - <i>C pneumoniae</i> IgG seropositivity .....	147
Table 6. Seroprevalences of <i>C pneumoniae</i> , <i>M pneumoniae</i> , and <i>L pneumophila</i> in control medical condition groups .....	149
Table 7. Seroprevalences of <i>C pneumoniae</i> , <i>M pneumoniae</i> , and <i>L pneumophila</i> IgG and IgM and aggregate number of pathogens in first-ever and recurrent stroke / TIA cases .....	151

## Chapter 8

Table 1. The difference in the mean IMD or IDAOPI deprivation scores between IgG / IgM seropositive and IgG / IgM seronegative or equivocal patients in stroke / TIA cases and medical controls .....	188
Table 2. The relationship between patients' mean IMD and IDAOPI deprivation scores and their seropositivity (IgG and IgM) for 0, 1, 2 or 3 of <i>C pneumoniae</i> , <i>M pneumoniae</i> , and <i>L pneumophila</i> .....	190

Table 3. The effect of IMD and IDAOPI deprivation scores on the odds ratios (ORs) for stroke / TIA in relation to IgG seropositivity for 0, 1, 2 or 3 of <i>C pneumoniae</i> , <i>M pneumoniae</i> , and <i>L pneumophila</i> .....	192
Table 4. The median survival (in months) of the stroke / TIA cases and medical controls as divided into 3 tertile groups of low, medium and high IMD or IDAOPI scores .....	194

## **List of published works submitted for / relevant to the thesis**

### **Book chapters**

1. **Ngeh J**, Gupta S. Inflammation and infection in coronary artery disease. In: Jackson G, ed. **Cardiology: Current Perspectives**. London: Martin Dunitz Ltd., 2002; 125-144.
2. **Ngeh J**, Gupta S. *Chlamydia pneumoniae* and atherosclerosis: an overview of the association. In: Herman Friedman, Yoshimasa Yamamoto, Mauro Bendinelli, eds. ***Chlamydia pneumoniae* Infection and Disease**. New York: Kluwer Academic / Plenum Publishers, 2004; 113-133.

### **Review papers**

1. **Ngeh J**, Gupta S. *Chlamydia pneumoniae* and atherosclerosis: causal or coincidental link? **ASM News (The News Magazine of the American Society for Microbiology)** 2000; 66 (12):732-737.
2. **Ngeh J**, Gupta S. Inflammation, infection and antimicrobial therapy in coronary heart disease- where do we currently stand? **Fundamental and Clinical Pharmacology** 2001;15:85-93.
3. **Ngeh J**, Anand V, Gupta S. *Chlamydia Pneumoniae* and atherosclerosis- what we know and what we don't. **Clinical Microbiology and Infection** 2002;8:2-13.

### **Research papers**

1. **Ngeh J**, Gupta S, Goodbourn C, Panayiotou B, McElligott G. *Chlamydia pneumoniae* in elderly patients with stroke (C-PEPS): a case-control study on the seroprevalence of *Chlamydia pneumoniae* in elderly patients with acute cerebrovascular disease. **Cerebrovascular Diseases** 2003;15:11-16.

2. **Ngeh J**, Gupta S, Goodbourn C. The reproducibility of an enzyme-linked immunosorbent assay (ELISA) used for detection of *Chlamydia pneumoniae*-specific antibodies. **Clinical Microbiology and Infection** 2004;10:171-174.
3. **Ngeh J**, Gupta S, Goodbourn C, McElligott G. *Mycoplasma pneumoniae* in elderly patients with stroke: a case-control study on the seroprevalence of *Mycoplasma pneumoniae* in elderly patients with acute cerebrovascular disease. The M-PEPS Study. **Cerebrovascular Diseases** 2004;17:314-319.
4. **Ngeh J**, Goodbourn C. *Chlamydia Pneumoniae*, *Mycoplasma Pneumoniae* and *Legionella Pneumophila* in Elderly Patients with Stroke (C-PEPS, M-PEPS, L-PEPS): a case-control study on the infectious burden of atypical respiratory pathogens in elderly patients with acute cerebrovascular disease. **Stroke** 2005;36:259-263.
5. **Ngeh J**, Goodbourn C. *Coxiella burnetii* and atypical respiratory infectious burden in stroke. **Stroke** 2005;36:1356.
6. **Ngeh JKT**. A case-control study on the prevalence of electrocardiographic rhythms and ischaemic changes in elderly patients with acute cerebrovascular disease. **American Journal of Geriatric Cardiology** 2004;13:248-251.
7. **Ngeh J**, Hackshaw A, Gupta S. The relationship between socioeconomic deprivation, atypical respiratory infections and survival outcome in elderly stroke and medical patients. **Cerebrovascular Diseases** 2007;24:546-547

## **Declaration of materials from a previously submitted dissertation**

### **Chapters 1 and 2**

These introductory chapters of the thesis contain materials from the following published paper and dissertation:

**Ngeh J**, Gupta S, Goodbourn C, Panayiotou B, McElligott G. *Chlamydia pneumoniae* in elderly patients with stroke (C-PEPS): a case-control study on the seroprevalence of *Chlamydia pneumoniae* in elderly patients with acute cerebrovascular disease. **Cerebrovascular Diseases 2003;15:11-16.**

As acknowledged in the above published paper, this study formed the basis of a dissertation submitted by myself in part fulfillment of the requirements of the Master of Science degree in Geriatric Medicine, in 2000:

**Ngeh J.** *Chlamydia Pneumoniae* in Elderly Patients with Stroke Study (CPEPS): A case-control study on the seroprevalence of *Chlamydia pneumoniae* in patients aged over 65 years admitted with acute stroke or transient ischaemic attack. **MSc Dissertation, University of Keele, UK, 2000.**

### **Chapter 7**

In this chapter, the prevalence of electrocardiographic rhythms and ischaemic changes of patients in the C-PEPS study were originally documented, but not analysed nor discussed in details, within the above MSc dissertation. Subsequent effort and case-control analysis of the electrocardiographic data resulted in the following publication that formed the basis of discussion in chapter 7 of this thesis:

**Ngeh JKT.** A case-control study on the prevalence of electrocardiographic rhythms and ischaemic changes in elderly patients with acute cerebrovascular disease. **American Journal of Geriatric Cardiology 2004;13:248-251.**



## **Declaration of published works done in collaboration with others**

### **Book chapters**

1. **Ngeh J**, Gupta S. Inflammation and infection in coronary artery disease. In: Jackson G, ed. **Cardiology: Current Perspectives. London: Martin Dunitz Ltd., 2002; 125-144.**
2. **Ngeh J**, Gupta S. *Chlamydia pneumoniae* and atherosclerosis- an overview of the association. In: Herman Friedman, Yoshimasa Yamamoto, Mauro Bendinelli, eds. ***Chlamydia pneumoniae* Infection and Disease. New York: Kluwer Academic / Plenum Publishers, 2004;113-133.**

Sandeep Gupta invited me to write these two book chapters with him as commissioned by the book editors. I performed an up-to-date literature search and review, and wrote the first drafts independently. S Gupta commented on my first drafts, and we were involved in subsequent drafting of the manuscripts. I obtained permission to reproduce some figures in the book chapters. I dealt with queries from the publishers directly. I proof read the manuscripts before its publication as book chapters.

### **Review papers**

1. **Ngeh J**, Gupta S. *Chlamydia pneumoniae* and atherosclerosis: causal or coincidental link? **ASM News (The News Magazine of the American Society for Microbiology) 2000; 66 (12):732-737.**
2. **Ngeh J**, Gupta S. Inflammation, infection and antimicrobial therapy in coronary heart disease- where do we currently stand? **Fundamental and Clinical Pharmacology 2001;15:85-93.**

**3. Ngeh J, Anand V, Gupta S. *Chlamydia Pneumoniae* and atherosclerosis- what we know and what we don't. *Clinical Microbiology and Infection* 2002;8:2-13.**

Sandeep Gupta invited me to co-author these review papers as commissioned by the journals. I performed the background literature review and wrote the first drafts independently. V Anand helped with some background literature search in one of the papers. All the authors were involved in subsequent drafting of the papers. I obtained permission to reproduce figures and tables in the papers. I answered queries raised by peer reviewers of the journals. I proof read the papers before its publication. S Gupta was the corresponding authors.

## **Research papers**

### **Paper 1**

**Ngeh J, Gupta S, Goodbourn C, Panayiotou B, McElligott G. *Chlamydia pneumoniae* in elderly patients with stroke (C-PEPS): a case-control study on the seroprevalence of *Chlamydia pneumoniae* in elderly patients with acute cerebrovascular disease. *Cerebrovascular Diseases* 2003;15:11-16.**

I initiated a literature search and review, and developed the research question independently. I wrote the first draft of the research protocol. All the co-authors commented / approved the final protocol. I obtained research approval from the hospital's medical director and Local Research Ethics Committee, with the support of G McElligott. I wrote a grant application, applied and obtained a research grant from the British Geriatrics Society. S Gupta obtained partial sponsorship of the commercial ELISA kits.

I calculated the study sample-size and recruited all the 187 patients prospectively. I obtained all the patients' sera and stored them in the laboratories. C Goodbourn and J Honeycombe (acknowledged in paper) performed the ELISA tests. I analysed and interpreted the results of the ELISA tests. I stored the patients' data and results of the ELISA tests in computer Microsoft Excel format. I liaised with A Hackshaw, a medical statistician who helped with statistical analyses of the research data.

I wrote the first draft of the paper independently. All the co-authors commented on subsequent drafts. I answered all the queries raised by peer-reviewers of the journal. I proof read the paper before its publication. I was the corresponding author.

## **Paper 2**

**Ngeh J**, Gupta S, Goodbourn C. The reproducibility of an enzyme-linked immunosorbent assay (ELISA) used for detection of *Chlamydia pneumoniae*-specific antibodies. **Clinical Microbiology and Infection** 2004;10:171-174.

I initiated a literature search and review, and asked the research question independently. S Gupta obtained partial sponsorship of the commercial ELISA kits. C Goodbourn and J Honeycombe (acknowledged in paper) performed the ELISA tests. I analysed and interpreted the results of the ELISA tests. I stored the patients' data and results of the ELISA tests in computer Microsoft Excel format. I liaised with A Hackshaw who performed statistical analyses of the research data.

I wrote the first draft of the paper. All the co-authors commented on my subsequent drafts. I answered all the queries raised by peer-reviewers of the journal. I proof read the paper before its publication. I was the corresponding author.

## **Paper 3**

**Ngeh J**, Gupta S, Goodbourn C, McElligott G. *Mycoplasma pneumoniae* in elderly patients with stroke: a case-control study on the seroprevalence of *Mycoplasma pneumoniae* in elderly patients with acute cerebrovascular disease. The M-PEPS study. **Cerebrovascular Diseases** 2004;17:314-319.

I initiated a literature search and review, and developed the research question independently. I wrote the first draft of the research protocol. All the co-authors commented / approved the final protocol. I obtained research approval from the Local Research Ethics Committee, with the support of G McElligott. S Gupta obtained partial sponsorship of the commercial ELISA kits.

C Goodbourn and J Honeycombe (acknowledged in paper) performed the ELISA tests. I analysed and interpreted the results of the ELISA tests. I stored the patients'

demographics and results of the ELISA tests in computer Microsoft Excel format. I liaised with A Hackshaw who performed statistical analyses of the research data.

I wrote the first draft of the paper. All the co-authors commented on subsequent drafts. I answered all the queries raised by peer-reviewers of the journal. I proof read the paper before its publication. I was the corresponding author.

#### **Paper 4**

**Ngeh J**, Goodbourn C. *Chlamydia pneumoniae*, *Mycoplasma pneumoniae*, and *Legionella pneumophila* in elderly patients with stroke (C-PEPS, M-PEPS, L-PEPS): a case-control study on the infectious burden of atypical respiratory pathogens in elderly patients with acute cerebrovascular disease. **Stroke** 2005;**36**:259-263.

I initiated a literature search and review, and developed the research question independently. I obtained research approval from the hospital's Research Ethics and Research and Development Committees. I wrote a grant application, applied and obtained a research grant from the British Geriatrics Society.

C Goodbourn and his colleagues performed the ELISA tests. I analysed and interpreted the results of the ELISA tests. I stored the patients' data and results of the ELISA tests in computer Microsoft Excel format. I liaised with A Hackshaw who helped with statistical analyses of the research data.

I wrote the first draft of the paper. C Goodbourn commented on my subsequent drafts. I answered all the queries raised by peer-reviewers of the journal. I proof read the paper before its publication. I was the corresponding author.

#### **Paper 5**

**Ngeh J**, Goodbourn C. *Coxiella burnetii* and atypical respiratory infectious burden in stroke. **Stroke** 2005;**36**:1356.

I initiated a literature search and review, and asked the research question independently. I obtained research approval from the hospital's Research Ethics and Research and Development Committees. I wrote a grant application, applied and

obtained a research grant from the British Geriatrics Society. C Goodbourn and his colleagues performed the ELISA tests. I analysed and interpreted the results of the ELISA tests. I stored the patients' data and results of the ELISA tests in computer Microsoft Excel format. I wrote the first draft of the paper. C Goodbourn commented on subsequent draft. I submitted and proof read the paper before its publication.

### **Paper 6**

**Ngeh JKT.** A case-control study on the prevalence of electrocardiographic rhythms and ischaemic changes in elderly patients with acute cerebrovascular disease. **American Journal of Geriatric Cardiology 2004;13:248-251.**

I was the sole author of this paper. I initiated a literature search and review, and asked the research question independently. I analysed and interpreted the electrocardiographic recordings of patients recruited in the earlier '*Chlamydia pneumoniae* in elderly patients with stroke' or 'C-PEPS' case-control study. I liaised with Dr Noel McCarthy, a medical statistician in Oxford who helped with statistical analyses of the data. I answered all the queries raised by peer-reviewers of the journal. I proof read the paper before its publication.

### **Paper 7**

**Ngeh J,** Hackshaw A, Gupta S. The relationship between socioeconomic deprivation, atypical respiratory infections and survival outcome in elderly stroke and medical patients. **Cerebrovascular Diseases 2007;24:546-547.**

I initiated literature search and review, and developed research questions independently. I collected individual patients' resident postcodes from the '*Chlamydia pneumoniae* in elderly patients with stroke' or C-PEPS case-control study database. S Gupta helped to verify these resident postcodes from the hospital's administrative records. I transformed these postcodes individually into English indices of socioeconomic deprivation scores (IMD 2004 and IDAOPI). I traced the patients' administrative and statutory records for any dates of death, and determined their survival outcome. I liaised with A Hackshaw who performed statistical analyses on the research data, and in conjunction with the C-PEPS, M-PEPS, and L-PEPS research database.

I wrote the first draft of the paper. A Hackshaw and S Gupta commented on my subsequent drafts. I answered all the queries raised by peer-reviewers of the journal. I proof read the paper before its publication. I was the corresponding author.

## **Acknowledgements**

I am most grateful to the British Geriatrics Society for the award of several Specialist Registrar Research Start-Up Grants in support of several research projects within this thesis.

I am grateful to Professor Michael Horrocks, my university supervisor, for his support, guidance and advice during the preparation and submission of this thesis.

I am greatly indebted to all my collaborators / co-authors who have contributed to the published works within this thesis:

Dr Colin Goodbourn for his expert advice and technical support on the microbiological aspects of the research projects, and for co-authoring several papers

Dr Sandeep Gupta for his expert advice, constructive criticisms and guidance during the C-PEPS, M-PEPS and several subsequent studies, and for co-authoring several papers including book chapters and review papers

Mr Allan Hackshaw for his expert statistical advice and analyses on the research data within the published works submitted for this thesis, and for co-authoring a paper

Dr Noel McCarthy for his expert statistical advice and analyses on the research data pertaining to the paper on the relationship between electrocardiography and acute cerebrovascular disease

Dr Laurence Gregory Wells for his helpful advice and discussions on the English indices of socioeconomic deprivation

Mrs Joyce Honeycombe for her technical assistance in ELISA serological analyses in the C-PEPS and M-PEPS studies

Dr Barnabas Panayiotou for his encouragement, constructive criticisms and guidance throughout the original C-PEPS study

Dr Geraldine McElligott for her support, guidance and permission to recruit her departmental colleagues' and her patients during the initial C-PEPS and M-PEPS studies

Dr V. Anand for co-authoring a review paper

Dr B. Chattopadhyay and Dr P. Foley for their kind permission to use their microbiology and chemical pathology laboratories during the initial C-PEPS study

I thank Savyon Diagnostics Limited, Israel, for supplying and partially sponsoring the SeroCP and SeroMP ELISA kits used in the C-PEPS, M-PEPS, and SeroCP ELISA reproducibility studies.

Last, but not least, I thank all the patients and their families or carers who agreed to participate in the clinical research.



## Abbreviations

A	Actin
AAA	Abdominal Aortic Aneurysm
ACADEMIC	Azithromycin in Coronary Artery Disease Elimination of Myocardial Infection with <i>Chlamydia</i>
ACES	Azithromycin and Coronary Events Study
ACS	Acute Coronary Syndrome
AV	Aortic Valve
AZACS	AZithromycin in Acute Coronary Syndromes
bFGF	basic Fibroblast Growth Factor
BU	Binding Units
CA	Carotid Atherosclerosis
CABG	Coronary Artery Bypass Graft
CAD	Coronary Artery Disease
CagA	Cytotoxin-Associated gene-A
C-BEPS	<i>Coxiella Burnetii</i> in Elderly Patients with Stroke
<i>C burnetii</i>	<i>Coxiella burnetii</i>
CHD	Coronary Heart Disease
CI	Confidence Interval
CLARICOR	CLARithromycin in patients with stable CORonary heart disease
CLARIFY	CLARithromycin in acute coronary syndrome patients in Finland
CMV	<i>CytoMegalovirus</i>
COI	Cut-Off Index
COV	Cut-Off Value
C-PEPS	<i>Chlamydia Pneumoniae</i> in Elderly Patients with Stroke
<i>C pneumoniae</i> / Cp	<i>Chlamydia pneumoniae</i>
CRP	C-Reactive Protein
CSF	Colony Stimulating Factor
CT	Computed Tomography
DIF	Direct genus-specific Immuno-Fluorescence staining
DNA	DeoxyriboNucleic Acid

EB	Elementary Body
ECG	ElectroCardioGraphy / ElectroCardioGraphic
ELISA	Enzyme-Linked ImmunoSorbent Assay
EM	Electron Microscopy
FMD	Flow-Mediated Dilation
GF	Growth Factor
GI	Gamma Interferon
HLA	Human Leucocyte Antigen
<i>H pylori</i>	<i>Helicobacter pylori</i>
HRP	HorseRadish Peroxidase
HSP	Heat Shock Proteins
ICAM	Inter-Cellular Adhesion Molecules
ICC	Immuno-Cyto-Chemistry
IDAOP1	Income Deprivation Affecting Older People Index
IFA	Indirect Fluorescence Antibody
Ig	Immunoglobulin
IHC	Immuno-Histo-Chemistry
IHD	Ischaemic Heart Disease
IL	InterLeukin
IMD	Index of Multiple Deprivation
IMT	Intima-to-Media Thickness
ISAR-3	Intracoronary Stenting and Antibiotic Regime trial-3
ISH	<i>In-Situ</i> Hybridisation
LDL	Low-Density Lipoprotein
L-PEPS	<i>Legionella Pneumophila</i> in Elderly Patients with Stroke
<i>L pneumophila</i> / Lp	<i>Legionella pneumophila</i>
LPS	LipoPolySaccharides
MAPK	Mitogen-Activated Protein Kinase
MCP-1	Monocyte Chemotactic Protein-1
MI	Myocardial Infarction
MIF	Micro-Immuno-Fluorescence
MIP	Macrophage Inflammatory Protein
MMP	Matrix MetalloProteinases
MOMP	Major Outer Membrane Proteins

M-PEPS	<i>Mycoplasma Pneumoniae</i> in Elderly Patients with Stroke
<i>M pneumoniae</i> / Mp	<i>Mycoplasma pneumoniae</i>
mRNA	Messenger RiboNucleic Acid
n	Number
NC	Negative Control
NF	Nuclear transcription Factors
NHS	National Health Service
NO	Nitric Oxide
No.	Number
NS	Non-Significant
OD	Optical Density
OR	Odds Ratio
P	Pseudopodia
PAD	Peripheral Arterial Disease
PB	Persistent Body
PC	ProstaCyclin
PCR	Polymerase Chain Reaction
PDGF	Platelet-Derived Growth Factor
PROVE-IT	PRavastatin Or atorVastatin Evaluation and Infection Therapy
PTCA	Percutaneous Transluminal Coronary Angioplasty
RB	Reticulate Body
RCT	Randomized Controlled Trial
ROXIS	ROXithromycin in Ischaemic Syndromes
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
SED	Socio-Economic Deprivation
SMC	Smooth Muscle Cell
SOA	Super Output Area
SPACE	Secondary Prevention of Atherosclerosis through <i>Chlamydia Pneumoniae</i> Eradication
STAMINA	South Thames trial of Antibiotics in Myocardial Infarction and uNstable Angina
TF	Tissue Factor
TIA	Transient Ischaemic Attack
TMB	TetraMethylBenzidine

TNF- $\alpha$	Tumour Necrosis Factor-Alpha
TWAR	TaiWan Acute Respiratory
UK	United Kingdom
V	Vacuoles
VCAM	Vascular Cell Adhesion Molecules
vs	versus
WIZARD	Weekly Intervention with Zithromax in Atherosclerosis-Related Disorders

## **Chapter 1**

**Infections as risk factor for stroke: focusing on the association between *Chlamydia pneumoniae* and atherosclerosis**

## **1.1. Introduction**

Stroke is defined clinically as ‘a syndrome of rapidly developing clinical signs of focal or global disturbance of cerebral function, with symptoms lasting 24 hours or longer or leading to death, with no apparent cause other than of vascular origin’. [World Health Organisation, 1978] Events lasting less than 24 hours are termed as transient ischaemic attacks (TIA).

Stroke is the third most common cause of death after coronary heart disease (CHD) and all cancers in the developed world. [Murray and Lopez, 1997] World Health Organisation data has revealed that world-wide 4.5 million people die each year due to stroke. [Al-Shahi, 2000] It is the commonest serious neurological problem [Hachinski, 1998] and a major cause of disability [Murray and Lopez, 1996] in the world.

In the United Kingdom, the incidence of first and recurrent stroke is around 2.4 per 1,000 people per year. [Wade, 1994] This rises to 3 per 1,000 per year among the 55-64-year-olds, and rapidly to 20 per 1,000 per year in people aged 85 years and over. The prevalence of stroke in the United Kingdom is 5-8 per 1,000 in people aged over 25. [Al-Shahi, 2000]

Stroke has been accounted for about 5% of the National Health Service (NHS) budget [Rudd et al, 1999] or £500 million (12%) of the NHS and Social Services budget [Al-Shahi, 2000]. It has been estimated that in England and Wales, there will be an increase of around 30% in the number of first-ever strokes between the years 1983-

2023. [Malmgren et al, 1989] With an ageing population, the burden of stroke in terms of mortality, morbidity and cost will continue to be enormous.

Stroke prevention will therefore continue to be the most effective strategy for reducing the adverse health and financial consequences of stroke. [Wolf, 1998] The identification and treatment of established stroke risk factors such as hypertension, cigarette smoking, diabetes mellitus, atrial fibrillation and hypercholesterolaemia, as well as the search and research for novel risk factors, are vital in the context of primary and secondary prevention strategies. [Warlow, 1998]

## **1.2. Pathological causes of stroke**

Stroke is a heterogeneous condition that may be due to arterial occlusion or haemorrhage. About 85% of strokes are occlusive. [Bamford, 1990] The occlusion of large vessels may be due to thrombosis or embolism, [Kalra, 1998] and accounts for approximately 70% of ischaemic stroke (50% from atherothromboembolism and 20% from cardiac embolism) [Warlow, 1996]. The occlusion of intracranial small vessels may give rise to lacunar stroke, which is associated with hypertension or embolism [Kalra, 1998] and causes about 25% [Warlow, 1996] of ischaemic strokes.

Haemorrhagic stroke accounts for about 15% of all strokes. The haemorrhage may be parenchymal due to Charcot-Bouchard microaneurysms (11 to 12%), or secondary to extracerebral rupture of intracranial vessels (berry aneurysms) in the subarachnoid space (3-4%). [Bamford, 1990] [Ebrahim, 1998] [Kalra, 1998]

A high index of suspicion of rare (about 5%) underlying causes of stroke is required, as they often require different management. [Anonymous, 1989], [Ebrahim, 1998] Examples of rare occlusive causes of stroke include arteritis, hyperviscosity syndromes, infections, atrial myxoma, arterial dissection, inherited collagen disorders. Haemorrhagic causes include bleeding diatheses, arteriovenous malformations, warfarin treatment, head injury, cerebral tumours and amphetamine abuse. Other miscellaneous rare causes of ischaemic stroke include congenital arterial anomalies and unruptured aneurysms, migraine, marantic endocarditis, malignant angioendotheliosis, the oral contraceptive pill and hormone replacement therapy, pregnancy, Moyamoya syndrome, mitochondrial cytopathy, cholesterol embolisation syndrome. [Warlow et al, 1996]

### **1.3. Acute infection and stroke**

Ischaemic stroke of undertermined aetiology represents approximately 40% of cases in stroke data banks, and the importance of potential precipitants or risk factors has not been well defined. [Sacco, 1989] Recent infection may be an underestimated risk factor for stroke. [Grau, 1995] For example, the increased rate of cardiovascular and stroke mortality in the winter months may partly be due to an increased prevalence of respiratory tract infections. [Woodhouse et al, 1994] It has been estimated that stroke occurs in almost 10% of bacteraemic cases without endocarditis [Svanbom, 1980] [Syrjanen, 1989], and about 10% of all strokes are associated with bacteraemic infections [Valtonen, 1993]. For the following discussion, acute infection is arbitrarily divided into systemic, central or local infection, although they often coexist clinically.



### **1.3.1. Acute systemic infection and stroke**

Acute infection is recognised as an important risk factor for ischaemic stroke. [Syrjanen et al, 1988] [Ameriso et al, 1991] [Grau et al, 1995] [Macko et al, 1996] [Bova et al, 1996] [Bornstein et al, 1997] [Grau et al, 1998] [Nencini et al, 2003] [Palm and Grau, 2007] In a case-control study involving patients aged under 50 years, a febrile infection during the preceding month was shown to be a significant and independent risk factor for ischaemic stroke [Relative Risk (RR)= 14.5; 95% Confidence Interval (CI)= 1.9 to 112]. [Syrjanen et al, 1988] In a larger case-control study involving patients aged 18 to 80 years, recent infection during the preceding week was found to be a significant risk factor for cerebral ischaemia, even when other risk factors for stroke were controlled for [Odds Ratio (OR)= 4.6; 95% CI= 1.9 to 11.3]. [Grau et al, 1995] Both of these studies revealed that infections among their patients were mainly of bacterial origin, and commonly affected the respiratory tract. Interestingly, a significant association of respiratory tract infection with the onset of myocardial infarction had also been reported. [Spodick et al, 1984]

A further case-control study suggested that both febrile and non-febrile infectious or inflammatory syndromes within the preceding one week were a common and important predisposing risk factor for brain infarction. [Macko et al, 1996] Using a control group consisting of consecutive patients who had suffered ischaemic stroke at least 6 months previously, a prospective, case-control study reported that acute infections of different types (mainly bacterial infection of the respiratory and urinary tracts) during the preceding week, among all age groups, constitute a significant risk factor for ischaemic stroke, even when other risk factors for stroke were adjusted for

(OR= 2.93; 95% CI= 1.64 to 5.26; P= 0.0002). [Bova et al, 1996] It was commented that recall bias or differences in motivation could not have explained the striking results. In another case-control study, the role of recent infection within the preceding week was found to be an independent risk factor for acute cerebrovascular ischaemia (adjusted OR= 3.1; 95% CI= 1.6 to 6.1), especially in the younger age groups. [Grau et al, 1998]

Recently, a very large UK study using within-person comparisons and case-series method confirmed that recent infection increased the risk of stroke and myocardial infarction. [Smeeth et al, 2004] Based on the United Kingdom General Practice Research Database, a total of 20,486 persons with a first myocardial infarction and 19,063 persons with a first stroke who received influenza vaccine were included in the analysis. The study showed that the risks of both vascular events were substantially increased after a diagnosis of systemic respiratory tract infection was made within the first three days (incidence ratio for stroke= 3.19; 95% CI= 2.81 to 3.62; incidence ratio for myocardial infarction= 4.95; 95% CI= 4.43 to 5.53) The risk of stroke was also increased within the first three days after diagnosis of urinary tract infection (incidence ratio= 2.72; 95% CI= 2.32 to 3.2). The risks then gradually fell during the following weeks after infection. In contrast, the study found no increase in the risk of stroke or myocardial infarction during the days or months after influenza, tetanus, or pneumococcal vaccination. [Smeeth et al, 2004] Indeed, influenza vaccination was associated with a reduction in the risk of stroke in two case-control studies [Lavalley et al, 2002] [Grau et al, 2005] and in one large observational study [Nichol et al, 2003]. [Palm and Grau, 2007]

The strong associations between recent respiratory infection and stroke or myocardial infarction were confirmed in a large case-control study recently [Clayton et al, 2008] Using a general practice database, the study identified 9,208 stroke cases and 11,155 myocardial infarction cases. The study found a strong evidence of an increased risk of both vascular events in the 7 days following respiratory infection: for stroke OR= 1.92 (95% CI= 1.24 to 2.97); for myocardial infarction OR= 2.10 (95% CI= 1.38 to 3.21). The investigators concluded that the benefits of reducing respiratory infection either through immunization or treating or preventing infection might be substantial. [Clayton et al, 2008]

### **1.3.2. Acute central or local infection and stroke**

Various bacterial, viral, fungal infections and worm infestations, affecting the meninges, carotid arteries, and endocardium have been described as causes of ischaemic stroke and TIA. [Warlow, 1996] Meningitis causes inflammation and secondary thrombosis of arteries and veins traversing the meninges to enter the brain. Therefore ischaemic events may be caused by acute bacterial meningitis due to organisms such as *Meningococcus*, *Pneumococcus*, *Haemophilus*, *Borrelia* and *Leptospira*. [Igarashi et al, 1984] Similarly acute tuberculous, syphilitic, or fungal (*Cryptococcus*, *Candida*, *Aspergillus*, *mucormycosis*) meningitis may cause sudden focal or multifocal ischaemic strokes. [Dalal and Dalal, 1989]

Some viruses such as *herpes zoster*, can cause periarterial inflammation and thrombosis. For example, middle cerebral artery occlusion with cerebral infarction has

been described a few weeks after the onset of ophthalmic zoster [Bourdette et al, 1983] [Sigal, 1987] and chickenpox (*Varicella zoster*) [Leopold, 1993].

Acquired immune deficiency syndrome (AIDS) caused by *Human immunodeficiency virus (HIV)* can be complicated by stroke. Although 40% of AIDS, AIDS-related complex, and *HIV* carriers have a neurological complication, only 1.3% has a stroke syndrome. [Pinto, 1996] Ischaemic infarcts are more common than intracerebral haemorrhages. [Pinto, 1996] Thrombocytopenia, primary central nervous system lymphoma, and metastatic Kaposi's sarcoma in AIDS may lead to intracranial haemorrhages. [Pinto, 1996] On the other hand, cerebral infarction can be caused by fungal meningitis, *Herpes zoster* and *HIV* vasculopathy, non-bacterial thrombotic or marantic endocarditis, and radiotherapy. [Park et al, 1990] [Kiebertz et al, 1993] [Pinto, 1996]

Worm infestation such as *neurotrichinosis* [Fourestie et al, 1993] and *cysticercosis* [Del Brutto, 1992], *mycoplasma* [Mulder and Spierings, 1987], *cat-scratch disease* [Selby and Walker, 1979] and tick-borne diseases e.g. Neuroborreliosis (*Borrelia burgdoferi*) [Reik, 1993], Rocky Mountains spotted fever (*Rickettsia rickettsiae*) [Grau et al, 2006], are all occasionally complicated by stroke.

Upper respiratory tract infection such as pharyngitis, tonsillitis and lymphadenitis, particularly in children, may be complicated by inflammation of the internal carotid artery in the neck, with secondary thrombosis. [Bickerstaff, 1964]

Infective causes of ischaemic stroke that are more prevalent in the developing than in the developed world include syphilis (*Treponema pallidum*), tuberculosis (*Mycobacterium tuberculosis*), cerebral embolism due to rheumatic heart disease or infective endocarditis, and malaria (*Plasmodium falciparum*). Parasitic infestations such as gnathostomiasis, schistosomiasis and larva migrans are recognised causes of intracerebral haemorrhage that are also more prevalent in the developing countries. [Poungvarin, 1998] Chagas disease (*Trypanosoma cruzi*) with embolism resulting from cardiomyopathy may lead to a stroke. [Grau et al, 2006]

### **1.3.3. Infective endocarditis and stroke**

Infective endocarditis is more common in the elderly, with more than 50% of patients having their first episode of endocarditis after the age of 60 years. [Von Reyn et al, 1981] Stroke, including cerebral infarctions due to cerebral artery thrombosis and emboli, and intracranial haemorrhages, is clinically the most common thrombo-embolic complication in patients with infective endocarditis. [Valtonen et al, 1993] About 20% of patients with endocarditis will develop a stroke during their illness. [Hart et al, 1990] Cerebral infarctions are about three or four times more common than intracranial haemorrhages, which are often due to ruptured mycotic aneurysms or pyogenic arteritis. [Hart et al, 1990] [Jones et al, 1969]

Apart from *Viridans streptococci*, [Grau et al, 2006] *Staphylococcus aureus* [Hart et al, 1990] and *Haemophilus parainfluenzae* [Chunn et al, 1977] [Lynn et al, 1977] endocarditis are predisposed to the development of thrombo-embolic complications.

However, intracranial haemorrhages may be more common in intravenous drug abusers due to *Staphylococcus aureus* endocarditis and associated pyogenic arteritis.

#### **1.4. Mechanisms of infection-associated stroke**

A procoagulant state is a link between infection and ischaemic stroke. [Grau et al, 1995] Infection may stimulate coagulation through inflammatory mechanisms such as the expression of thromboplastin by monocytes and macrophages. [Rivers et al, 1975] With an increased serum levels of tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) and other cytokines, [Van der Poll et al, 1990] [Schleef et al, 1988] the coagulant function of the endothelium may be altered to express tissue factor, increased levels of plasminogen activator inhibitor (PAI-1) and reduced levels of tissue plasminogen activator, [Palm and Grau, 2007], inhibition of the protein C / protein S anticoagulant system, [Esmon et al, 1991] [Hesseltvik et al, 1991] and leading to increased levels of clotting factors such as fibrinogen and Factor VII [Woodhouse et al, 1994]. Indeed, stroke patients with a recent infection were found to have the lowest concentrations of activated protein C, elevated levels of C4b-binding protein which binds protein S (an important cofactor of protein C), and a lower ratio of active tissue plasminogen activator to PAI. [Macko et al, 1996(b)]

Patients with infection-associated ischaemic stroke have significantly increased fibrin D-dimer generation, increased cardiolipin immunoreactivity, and hyperfibrinogenemia, when compared with stroke patients without infections. [Ameriso et al, 1991] Antithrombin III and protein C and S deficiency, increased platelet activation, aggregation and adhesion, impairment of endogenous fibrinolysis

as indicated by reduced tissue plasminogen activator (TPA) activity, and a lower ratio of active TPA to plasminogen activator inhibitor-1, may contribute to the increased risk for brain infarction after recent infection / inflammation. [Bone 1992] [Macko et al, 1996] [Palm and Grau, 2007] Antiphospholipid antibodies formed in septic patients may interact with protein C and S, leading to thrombo-embolic complications such as stroke. [McNeil et al, 1991] [Parke AL et al, 1992] [Macko et al, 1996]

Infection is known to activate leucocytes, which in turn interact with platelets. [Marcus et al, 1988] [Vasthare et al, 1990] [Galante et al, 1992] [Carlos and Harlan, 1994] [Grau et al, 1995] [Elneihoum et al, 1996] Leucocytes e.g. monocytes may be induced to produce tissue factor, secrete cytokines such as TNF- $\alpha$ , interleukin-1 $\beta$ , interleukin-6, and interferon- $\gamma$ , and others that have a procoagulant effect, resulting in thrombotic events. [Passbender et al, 1994] [Barone et al, 1997] [Garcia et al, 1995] [Elkind and Cole, 2006] Higher levels of C-reactive protein that can induce increased tissue factor expression by monocytes, were also found in infection-associated stroke. [Grau et al, 1998] Therefore, inflammation may lead to endothelial dysfunction, linking infection and risk of an acute cardiovascular event. [Hingorani et al, 2000] [Vallance et al, 1997] [Palm and Grau, 2007]

In addition to activation of the coagulation system, septic emboli, cerebral vasculitis, meningitis, and spasms of arteries, can all lead to thrombosis of cerebral arteries and ischaemic stroke in patients with infective endocarditis. [Valtonen et al, 1993] [Grau et al, 1995] Although stroke in septic patients is infrequent, mental changes or so-called septic encephalopathy is the most common central nervous system

complication in patients with bacteraemia. [Syrjanen, 1989] Acute infection, although underestimated, is undoubtedly an important and significant risk factor of stroke.

## **1.5. Pathogenesis of atherosclerosis**

### **1.5.1. Definition of atherosclerosis**

Atherosclerosis is a disease of the blood vessels consisting of both degenerative and regenerative processes that initially affect the intima and at a later stage the media at the bifurcations of the major arteries. [Tegos et al, 2001] The term atherosclerosis is derived from the Greek words 'athero' and 'sclerosis'. 'Athero' means gruel and corresponds to the necrotic core at the base of the atherosclerotic plaque, whereas 'sclerosis' means hardening or induration, and corresponds to the fibrotic cap at the luminal edge of the plaque. [Tegos et al, 2001] The three main components of atherosclerotic lesions are: 1. cholesterol esters; 2. smooth muscle cells, macrophages and other cell types; and 3. connective tissue composed of collagen, elastin and glycosaminoglycans. [Woolf et al, 1990] [Davies and Woolf, 1993] [Tegos et al, 2001]

### **1.5.2. Theories of atherogenesis**

The five theories of atherogenesis are: 1. the lipid theory; 2. the haemodynamic theory; 3. the fibrin incrustation theory; 4. the nonspecific mesenchymal hypothesis; and 5. the response to injury hypothesis. [Tegos et al, 2001]

The lipid theory has established that earlier atherosclerotic lesions i.e. fatty streaks, are characterized by the accumulation of cholesterol esters within the macrophage



foam cells. [Stryer et al, 1994] [McGill HC Jr, 1968] [Tegos et al, 2001] Subsequent atherosclerotic plaque is characterised by the accumulation of cholesterol esters in the extracellular space. [Stryer et al, 1994] [Stryer et al, 1995] [Tegos et al, 2001] The accumulation of LDL-cholesterol in the intima could be due to: 1. increased plasma LDL concentrations; [Smith, 1990] 2. alteration of the arterial intima permeability to LDL, [Zhang et al, 1993] mediated via vesicular transport [Smith, 1990]; 3. increased LDL retention in the intima [Bondjers et al, 1990]; and 4. impeded LDL transport from the intima to the media. [Smith, 1990]

The haemodynamic theory of atherogenesis first proposed in 1950 suggested that hydrostatic and shear forces are responsible for the development and focal nature of atherosclerotic lesions. [Tegos et al, 2001] Hypertension may therefore predispose to the development of atherosclerotic lesions which have a predilection for the branching sites of the arterial system whereby turbulence, oscillating or low shear stress is usually detected. [Tegos et al, 2001] As a result of altered haemodynamics, the clearance of blood components may be delayed, thereby allowing prolonged contact of potentially toxic substances with the intima. [Tegos et al, 2001] This could potentiate endothelial injury, modify endothelial permeability and facilitate LDL transport to the intima. [Davies et al, 1984] [Ku et al, 1985] [Glagov et al, 1988] [Tegos et al, 2001]

The fibrin incrustation theory was first proposed by Rokitansky, a nineteenth century pathologist. [Rokitansky, 1952] [Tegos et al, 2001] It was postulated that fibrinogen is converted into fibrin with the formation of a thrombus on the arterial luminal surface. Duguid [Duguid, 1946] and Hand [Hand and Chandler, 1962] independently

demonstrated thrombus formation on plaque surface and its subsequent incorporation and transformation into fibrous tissue over time, thus increasing the plaque's size. [Tegos et al, 2001] They also proposed the phagocytosis of cholesterol-rich platelets by macrophages leading to the production of lipid-rich foam cells. [Tegos et al, 2001] However, it was commented that the layered appearance of the atheroma is more easily explainable on the basis of smooth muscle hyperplasia and connective tissue deposition rather than on the organisation of the thrombus. [Tegos et al, 2001] Nevertheless, this theory proposed the idea that thrombus formation in a complicated atherosclerotic plaque can lead to its instability. [Badimon et al, 1992] [Tegos et al, 2001]

The non-specific mesenchymal hypothesis proposed that a wide range of stimuli to the arterial wall, such as shear stress or vasoactive agents, may induce a migration of smooth muscle cells (mesenchymal cells) from the media to the intima, and which may subsequently proliferate and produce connective tissue. [Haus, 1962] [Tegos et al, 2001] The main components of this connective tissue are: 1. proteoglycans, which may trap the infiltrating LDL; and 2. collagen, which is the main space-filling agent of the atherosclerotic plaque. [Berenson et al, 1984] [Tegos et al, 2001] Indeed, the proliferation of smooth muscle cells and connective tissue production represent the regenerative process of atherosclerosis, which resembles the healing process in many aspects. [Tegos et al, 2001]

The 'response to injury' hypothesis was proposed in the 1970s. [Ross and Glomset, 1973] The hypothesis was progressively modified with increasing knowledge on the cellular and molecular biology of the arterial wall, the interaction between the cells of

the arterial wall and blood circulation, the role of vascular risk factors and endothelial cell damage. [Ross, 1993] According to the hypothesis, a number of factors may cause injury to the endothelial cells at specific sites in the artery. [Mora et al, 1987] [Tegos et al, 2001] These factors include hypertension, LDL-cholesterol, toxins in cigarette smoking, glycosylated end-products (diabetes mellitus), immune complexes and infection. [Fuster et al, 1992] [Tegos et al, 2001] Endothelial cell damage caused by these factors may lead to cellular activation and expression of binding sites for circulating monocytes via adhesion molecules e.g. vascular cell adhesion molecule-1 (V-CAM-1) and monocyte chemotactic agents e.g. monocyte chemotactic protein-1 (MCP-1). [Valente et al, 1992] [Tegos et al, 2001] With an increased vascular permeability, the monocytes and T lymphocytes attached to the endothelium migrate into the intima; and the monocytes then transform into tissue macrophages. [Gerrity RG, 1981] [Taylor and Lewis, 1986] [Munro and Cotran, 1988] [Hansson et al, 1989(b)] [Tegos et al, 2001] The plasma LDL-cholesterol may also traverse the endothelium, accumulate in the interstitial space of the intima, and form complexes with extracellular tissue collagen, elastin and proteoglycans. [Berenson et al, 1984] [Tegos et al, 2001] Oxidised LDL-cholesterol within the subendothelial space may also be internalised by the tissue macrophages (via the scavenger receptor) which become lipid-laden and transform into foam cells. [Jurgens et al, 1987] [Steinberg et al, 1989] [Tegos et al, 2001] Together with the lymphocytes, these foam cells that make up the fatty streak are the pathological hallmarks of early atherosclerotic lesions. [Faggiotto et al, 1984] [Rosenfeld et al, 1987] [Masuda and Ross, 1990] [Stary et al, 1994]. In addition, the platelets adhered to the site of endothelial injury may release platelet-derived growth factor which in turn may induce the migration of smooth muscle cells from the media to the intima. [Tegos et al, 2001] These smooth muscle

cells may proliferate and produce connective tissue such as collagen, elastin and proteoglycans. [Clowes et al, 1989] [Tegos et al, 2001] These smooth muscle cells and extracellular matrix ultimately lead to the genesis of fibrous tissue and calcification, forming an advanced atherosclerotic plaque. [Stary et al, 1995] However, the activated T lymphocytes found in the fibrous cap of the atherosclerotic lesions may be stimulated by oxidised LDL-cholesterol to produce  $\gamma$ -interferon which may inhibit smooth muscle proliferation, collagen and elastin synthesis, and decrease cholesterol accumulation. [Munro and Cotran, 1988] [Hansson et al, 1989(a)] [Hansson et al, 1989(b)] [O'Brien, 1994] [Stary et al, 1995] [Tegos et al, 2001] The interactions of macrophages, smooth muscle cells and T lymphocytes are therefore important in the synthesis of the extracellular matrix and plaque remodeling as the atherosclerotic lesion progresses. [Tegos et al, 2001]

## **1.6. Chronic infection and atherosclerosis or stroke**

The idea that chronic infection could play an important role in atherosclerosis is not entirely new. In fact the concept of an 'infectious' hypothesis of atherosclerosis was proposed by several prominent historical figures such as Virchow in 1859 and Osler in 1908, followed by Frothingham in 1911, and Ophuls in 1921. [Ngeh and Gupta, 2002] Indeed, over 20 specific microorganisms (bacteria and viruses) / antigens have been named to associate infection and atherosclerosis (see Table 1). [Nieto, 1998] [Ngeh and Gupta, 2004] [Xu et al, 1993] [Mayr et al, 1999] [2005, Fiehn et al] Among these, *Cytomegalovirus* (CMV), *C pneumoniae*, *Helicobacter pylori*, and dental (periodontal) pathogens are the micro-organisms most commonly studied. [Fong, 2002] [Leinonen and Saikku, 2002] [Ngeh and Gupta, 2004]

**Table 1. Microorganisms implicated to associate infections with atherosclerotic vascular diseases [Ngeh and Gupta, 2004]**

<b><u>Microorganism</u></b>	<b><u>Year and author of publication</u></b>
<i>Bacillus typhosus</i>	1889, Gilbert and Lion
<i>Streptococci</i>	1931, Benson et al
<i>Coxsackie B virus</i>	1968, Sohal et al
<i>Adenovirus</i>	1973, Fabricant et al
<i>Mycoplasma gallisepticum</i>	1973, Clyde and Thomas
<i>Marek's disease virus</i>	1978, Fabricant et al
<i>Cytomegalovirus</i>	1987, Petrie et al
<i>Herpes simplex virus</i>	1987, Hajjar et al
<i>Chlamydia pneumoniae</i>	1988, Saikku et al
<i>Measles virus</i>	1990, Csonka et al
<i>Epstein-Barr virus</i>	1993, Straka et al
<i>Human immunodeficiency virus</i>	1993, Paton et al
<i>Mycobacteria (hsp65)</i>	[1993, Xu et al]
<i>Helicobacter pylori</i>	1994, Mendall et al
<i>Mycoplasma fermentans</i>	1996, Ong et al
<i>Enteroviruses</i>	1998, Roivainen et al
<i>Coxiella burnetti</i>	1999, Lovey et al
<i>Escherichia coli</i>	[1999, Mayr et al]
<i>Porphyromonas gingivalis</i>	1999, Chiu et al
<i>Streptococcus sanguis</i>	1999, Chiu et al
<i>Actinobacillus actinomycetemcomitans</i>	2000, Haraszthy et al
<i>Bacteroides forsythus</i>	2000, Haraszthy et al
<i>Hepatitis A virus</i>	2000, Zhu et al
<i>Influenza virus</i>	2000, Naghavi et al
<i>Mycoplasma pneumoniae</i>	2000, Higuchi et al
<i>Prevotella intermedia</i>	2000, Haraszthy et al
<i>Varicella-zoster virus</i>	2000, Moriuchi and Rodriguez
<i>Hepatitis C virus</i>	[2002(a), Ishizaka et al]
<i>Hepatitis B virus</i>	[2002(b), Ishizaka et al]
<i>Campylobacter rectus</i>	[2005, Fiehn et al]

### 1.6.1. Cytomegalovirus

Cytomegalovirus is ubiquitously present and it was estimated that 60-90% of adults have been infected with this virus. [Bruggeman, 1993] [Palm and Grau, 2007] *CMV* infections can present as an acute febrile illness although they are often asymptomatic. [Palam and Grau, 2007] *CMV* has been associated with post-transplant vasculopathy in heart transplant patients. [Ventura et al, 1995] [Elkind and Cole, 2006] Serological evidence of *CMV* infection has also been found more commonly in patients with CHD than normal controls. [Melnick et al, 1990] Elevated levels of *CMV* antibody titres were also found to associate with an increased risk of carotid-intimal thickening 13-18 years later, [Nieto et al, 1996] [Palm and Grau, 2007] and atherosclerotic carotid stenosis [Espinola-Klein et al, 2000] [Elkind and Cole, 2006]

Prospective studies have also shown that subjects in the upper quintile of *CMV* titres have twice the risk of developing CHD than those in the lowest. [Sorlie et al, 2000] [Elkind and Cole, 2006] Among those with CHD, elevated *CMV* titres, as well as titres for hepatitis A virus and herpes simplex virus-2, were found to be associated with an increased risk of future myocardial infarction. [Muhlestein et al, 2000] [Elkind and Cole, 2006] Other studies have found that restenosis after coronary angiography occurs more frequently in patients positive for *CMV*. [Speir et al, 1994] [Elkind and Cole, 2006] Indeed, *CMV* has been detected by polymerase chain reaction (PCR) techniques in atherosclerotic lesions of those with CHD more frequently than in those without atherosclerosis. [Hendrix et al, 1990] [Elkind and Cole, 2006] However, two prospective studies have not confirmed the association between

seropositivity for *CMV* and future risk of stroke and cardiovascular events. [Ridker et al, 1998] [Fagerberg et al, 1999] [Palm and Grau, 2007]

*CMV* infection might promote atherogenesis through a number of potential mechanisms. [Elkind and Cole, 2006] [Palm and Grau, 2007] These include disrupting cell cycle control in vascular endothelial and smooth muscle cells [Speir et al, 1994] [Murphy et al, 2000], increasing procoagulant proteins [van Dam-Mieras et al, 1992], reactive oxygen species [Shibutani et al, 1997] [Speir et al, 1996] [Speir et al, 1998] and leucocyte adhesion molecules [Sindre et al, 2000]. Other mechanisms include an increased expression of proinflammatory cytokines such as interleukin-2 and interleukin-2 receptor [Geist et al, 1991], tumour necrosis factor-alpha [Geist et al, 1994] and nuclear factor-kappa B (a pro-inflammatory transcription factor) [Kowalik et al, 1993]; expression of platelet-derived growth factor and transforming growth factor [Lemstrom et al, 1994]; and smooth muscle cell proliferation and migration [Yonemitsu et al, 1997]. Although most of these mechanisms have been demonstrated in animal models, *CMV* infection has not been fully established as a risk factor for atherosclerosis and stroke. [Palm and Grau, 2007]

### **1.6.2. *Helicobacter pylori***

*Helicobacter pylori* are Gram-negative spiral bacteria. Infection begins in childhood and is influenced by socioeconomic factors. [Graham et al, 1991] [Mendall et al, 1992] [Palm and Grau, 2007] *H. pylori* may cause gastritis, peptic ulcers, non-ulcer dyspepsia and gastric cancers, but often remain asymptomatic. [Veldhuyzen van Zanten et al, 1994]

Retrospective seroepidemiological investigations have found an association between *H pylori* infection and atherosclerosis, [Danesh et al, 1997] although prospective studies that adjusted for other risk factors have not confirmed an association [Koenig et al, 1999]. [Elkind and Cole, 2006] Some studies have shown a correlation between *H pylori* seropositivity and risk of atherothrombotic or lacunar stroke, [Markus and Mendall, 1998] [Heuschmann et al, 2001] [Grau et al, 2001] [Ponzetto et al, 2002] but others did not [Whincup et al, 1996]. [Palm and Grau, 2007] In a recent case-control study, chronic *H pylori* infection was found to be associated with acute ischaemic stroke (OR= 2.57; 95% CI= 1.09 to 6.08] due to large and small artery diseases, but not cardiogenic embolism. [Sawayama et al, 2005]

Some studies have found that certain virulent *H pylori* strains bearing cytotoxin-associated gene-A (CagA), [Pasceri et al, 1998] can increase cytokine expression and are associated with increased inflammation. [Elkind and Cole, 2006] [Grau et al, 2006] [Palm and Grau, 2007] Seroepidemiological case-control studies of *H pylori* CagA strains were found to be significantly associated with stroke risk after adjustment for parental social class and other potential confounders. [Pietroiusti et al, 2002] [Preusch et al, 2004] [Palm and Grau, 2007] A meta-analysis including seven studies have reported an odds ratio of 1.49 (95% CI: 1.24-1.81) for the association between *H pylori* seropositivity and stroke, and an odds ratio of 2.23 (95% CI: 1.49-3.36) for the association between anti-CagA seropositivity and stroke. [Cremonini et al, 2004] [Palm and Grau, 2007] Furthermore, infection with CagA positive *H pylori* strains in atherosclerotic stroke patients has been found to associate with greater intima-media



thickness and poorer short-term outcome compared with CagA negative patients. [Diomedes et al, 2004]

Indeed, *H pylori* have also been identified directly in carotid plaques. [Ameriso et al, 2001] Postulated mechanisms linking *H pylori* infection and stroke include molecular mimicry leading to a cross-reaction between antibodies against CagA and vascular wall antigens. [Palm and Grau, 2007] Others have postulated that chronic *H pylori* infection would increase the pH level of gastric juice and decrease ascorbic acid level. This would result in a reduction of folate absorption which could hamper methionine synthase reaction. [Corrado and Novo, 2005] This may lead to an increased homocysteine level in the blood that may contribute to endothelial cell damage and atherosclerosis. [Corrado and Novo, 2005] However, a causal relationship between *H pylori* infection and atherosclerosis or stroke has not been proven conclusively. [Palm and Grau, 2007]

### **1.6.3. Periodontal pathogens**

Periodontal disease is a chronic progressive infection of the gingival, supporting connective tissue, and alveolar bone that hold the teeth in place. [Elkind and Cole, 2006] There are over 300 types of bacteria that live in the oral cavity, but only a minority of these, predominantly gram-negative micro-organisms, is associated with gingivitis and periodontitis. [Elkind and Cole, 2006] Some more important examples of these micro-organisms are *Porphyromonas gingivalis*, *Bacteroides intermedius*, *Actinobacillus actinomycetemcomitans*, *Bacteroides forsythus*, *Selenomonas sputigena*, *Eikenella corrodens*, *Fusobacterium nucleatum*, *Peptostreptococcus*

*anaerobius*, *Haemophilus wolinella*, *Prevotella intermedia*, *Campylobacter rectus*, *Tannerella forsythensis*, oral streptococci and spirochetal species. [Williams, 1990] [Elkind and Cole, 2006] [Grau et al, 2006] A prospective seroepidemiological study has observed an association between two major periodontal pathogens and future stroke. [Pussinen et al, 2004] [Palm and Grau, 2007] Several periodontal pathogens including *Porphyromonas gingivalis*, *Prevotella intermedia*, *Campylobacter rectus*, *Actinobacillus actinomycetemcomitans*, *Tannerella forsythensis*, and oral streptococci have also been identified in carotid atherosclerotic plaques [Chiu, 1999] [Fiehn et al, 2005] [Grau et al, 2006], although this has not been confirmed in another study [Aimetti et al, 2007]. However, experimental animal model has demonstrated that chronic inoculation of *P. gingivalis* could worsen lipid profiles, enhance atheroma formation and produce calcification of aortic atherosclerotic plaques. [Li et al, 2002] [Palm and Grau, 2007]

An association between tooth loss, periodontitis and carotid artery plaque prevalence has been observed. [Desvarieux et al, 2004] [Palm and Grau, 2007] Cross-sectional studies have demonstrated a correlation between dental radiograph abnormalities and carotid plaque. [Engebretson et al, 2005] [Elkind and Cole, 2006] Periodontal bone loss scores have also been found to be associated with stroke (adjusted odds ratio= 2.8), more significantly than total coronary heart disease (OR= 1.5) and fatal coronary heart disease (OR= 1.9). [Beck et al, 1996] [Elkind and Cole, 2006] Other studies that have adjusted for confounding factors, have found an association between periodontal disease and stroke (relative risk= 2.1), [Wu et al, 2000] but not for coronary heart disease [Hujoel et al, 2000]. [Elkind and Cole, 2006] A more recent case-control study has also found an association between severe periodontitis (mean attachment

loss > 6 mm) and stroke / TIA, independent of traditional risk factors (odds ratio= 4.3, 95% CI= 1.85-10.2). [Grau et al, 2004] [Palm and Grau, 2007]

The periodontal pathogens may enter into the blood stream during minor dental manipulations including chewing, brushing and flossing. [Silver et al, 1977] Bacteremia and secondary infection of heart valves with embolism to the brain may cause a stroke. [Elkind and Cole, 2006] These micro-organisms can produce lipopolysaccharide that activates macrophages and contributes to the production of proinflammatory cytokines such as prostaglandins E2, interleukin-1 $\beta$ , and TNF- $\alpha$ . [Williams, 1990] These cytokines may also contribute to a pro-coagulant state through activation of endothelial cells and platelets, and an increased production of fibrinogen and von Willebrand factor. [Elkind and Cole, 2006] Indeed, in prospective interventional studies, a reduction in serum inflammatory markers (C-reactive protein and interleukin-6) and blood pressure, and an improvement in lipid profiles after intensive periodontal therapy have been demonstrated. [D'Aiuto et al, 2004(a)] [D'Aiuto et al, 2004(b)] [D'Aiuto et al, 2005] [D'Aiuto et al, 2006] [Palm and Grau 2007] However, definitive evidence of a causal relationship between periodontal disease and stroke has not been established. [Palm and Grau, 2007]

## **1.7. *Chlamydia pneumoniae***

### **1.7.1. Introduction**

By far, the evidence linking *Chlamydia pneumoniae* infection and atherosclerosis or stroke is most abundant and appears to be the strongest. [Ngeh et al, 2002] [Ngeh and

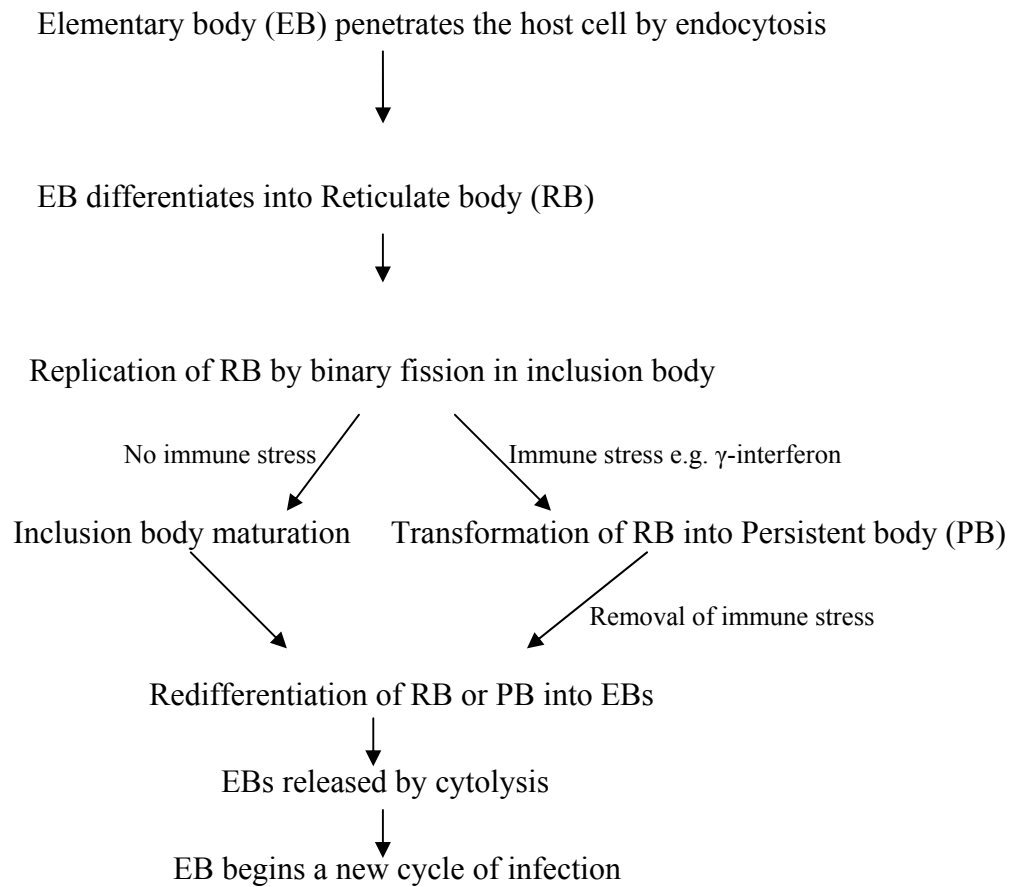
Gupta, 2004] *C pneumoniae* was first isolated from the conjunctiva of a child in Taiwan in 1965 and designated as TW-183. [Dugan et al, 2002] [Ngeh and Gupta, 2004] In 1983, it was isolated from the respiratory tract of a university student suffering from pharyngitis in Seattle, and designated as AR-39. [Dugan et al, 2002] [Ngeh and Gupta, 2004] *C pneumoniae*, thought to be a new strain of *Chlamydia psittaci* causing acute respiratory tract infection, was described by Grayston and his colleagues in 1986, and given the acronym TWAR (TaiWan Acute Respiratory). [Ngeh and Gupta, 2004] However, it was Saikku and his colleagues who first suggested that *Chlamydia* TWAR was associated with CHD or atherosclerosis in 1988. [Leinonen and Saikku, 2002] TWAR was subsequently renamed as *Chlamydia pneumoniae*, and recognised as a separate species in the genus *Chlamydia* in 1989. [Ngeh and Gupta, 2004]

### **1.7.2. Microbiology and epidemiology of *Chlamydia pneumoniae***

Apart from *Chlamydia trachomatis* and *Chlamydia psittaci*, *C pneumoniae* is the only other species that causes human diseases within the genus of *Chlamydia*. [Ngeh and Gupta, 2004] *C pneumoniae* is a gram-negative, obligate intracellular bacterium that depends on its host cell for growth and survival. [Kalayoglu et al, 2002] The outer membrane of *C pneumoniae* is composed of lipopolysaccharides (LPS) and heat-shock proteins (HSP) that are genus specific. [Ngeh and Gupta, 2004] However, several major outer membrane proteins (MOMP) identified are detectable by monoclonal antibodies and are species specific. [Ngeh and Gupta, 2004]

*Chlamydia pneumoniae* exists in three forms in its unique life cycle: 1. infectious, non-replicating elementary body (EB), 2. non-infectious but actively replicating reticulate body (RB), and 3. non-infectious, non-replicating persistent body (PB) (see Figure 1). [Kalayoglu et al, 2002] [Dugan et al, 2002] [Ngeh and Gupta, 2004] For example, the EB spore attacks the host's cell by endocytosis and differentiates into RBs by binary fission in enlarging vacuoles called inclusion bodies. [Kalayoglu et al, 2002] [Dugan et al, 2002] [Ngeh and Gupta, 2004] After inclusion maturation, the RBs redifferentiate into EBs, which are released by cytolysis, and start another cycle of infection. [Kalayoglu et al, 2002] [Dugan et al, 2002] [Ngeh and Gupta, 2004] However, the EB may transform into PB under conditions of immune stress such as in the presence of gamma interferon. [Kalayoglu et al, 2002] [Ngeh and Gupta, 2004] The PB may remain metabolically inactive and undetected by the host's immune system, and remain unresponsive to antibiotics. [Kalayoglu et al, 2002] [Ngeh and Gupta, 2004] When the stress is removed, the PB may redifferentiate into infectious EB and released by cell lysis to begin a new life cycle. (see Figure 1) [Kalayoglu et al, 2002] [Ngeh and Gupta, 2004]

**Figure 1. Life cycle of *Chlamydia pneumoniae***



Clinically, *C pneumoniae* is primarily a respiratory pathogen, although it also contributes to extra-pulmonary manifestations. [Kalayoglu et al, 2002] [Leinonen and Saikku, 2002] [Ngeh and Gupta, 2004] The infection is common worldwide and accounts for 5-20% of all cases of community-acquired pneumonia. [Ngeh and Gupta, 2004] However, *C pneumoniae* infection can also be asymptomatic. [Dugan et al, 2002] The incubation period of the infection is around 3-4 weeks. [Ngeh and Gupta, 2004] Cyclical epidemics due to *C pneumoniae* have been observed to occur every 4-7 years in western European countries. [Ngeh and Gupta, 2004] Most people are

infected several times in their lifetime. [Dugan et al, 2002] [Ngeh and Gupta, 2004]  
The seroprevalence of *C pneumoniae* is about 50% in middle-aged adults and rises to 70%-80% in older populations. [Ngeh and Gupta, 2004] [Higgins, 2003] [Kalayoglu et al, 2002] [Ngeh, 2000]

The evidence linking *C pneumoniae* infection and atherosclerosis is derived from five areas of research: seroepidemiological, pathological, animal, immunological and antibiotic treatment studies. [Dugan et al, 2002] [Fong, 2002] [Kalayoglu et al, 2002] [Leinonen and Saikku, 2002] [Higgins, 2003] [Ngeh and Gupta, 2004]

### **1.7.3. Evidence linking *Chlamydia pneumoniae* and atherosclerosis:**

#### **1.7.3.1. Seroepidemiological observations**

Seroepidemiological studies are credited as the earliest area of research linking *C pneumoniae* and atherosclerotic vascular diseases. Since Saikku and his colleagues first showed a positive association between CHD and *C pneumoniae* antibodies in a case-control study in 1988, numerous (n> 100) sero-epidemiological studies of various designs i.e. retrospective, cross-sectional, case-control, or prospective studies, have suggested a positive association. [Ngeh and Gupta, 2004] More recently, seroepidemiological studies have also showed a positive association between *C pneumoniae* serological markers and other atherosclerotic vascular diseases such as stroke, abdominal aortic aneurysm, and peripheral arterial diseases. [Dugan et al, 2002] [Ngeh et al, 2002] [Ngeh and Gupta, 2004]

Most of these positive seroepidemiological studies reported odds ratios (ORs) of 2 or higher. [Kalayoglu et al, 2002] [Higgins, 2003] [Ngeh and Gupta, 2004] However, a number of negative studies have also emerged. [Dugan et al, 2002] [Fong, 2002] [Kalayoglu et al, 2002] [Higgins, 2003] [Ngeh et al, 2002] [Ngeh and Gupta, 2004] More recently, large-scale prospective seroepidemiological studies and meta-analyses involving over 5000 cases have suggested a modest or weak association between *C pneumoniae* serological markers and CHD, with ORs between 1.15 to 1.25. [Kalayoglu et al, 2002] [Fong, 2002] [Ngeh and Gupta, 2004] Although some studies do not fully adjust for the influence of risk factors associated with *C pneumoniae* infection, others have been criticised for over-adjustment. [Fong, 2002]

The current gold standard in *C pneumoniae* serology is microimmunofluorescence (MIF), a test originally developed to diagnose clinical *C pneumoniae* infection, rather than for epidemiological study of the role of *C pneumoniae* in atherosclerosis. [Leinonen and Saikku, 2002] The MIF is a subjective test and is dependent on an experienced microscopist. [Ngeh and Gupta, 2004] Different seroepidemiological studies have used different cut-off titres making comparison of data difficult. [Kalayoglu et al, 2002] [Higgins, 2003] [Ngeh and Gupta, 2004] Further efforts are required to standardise *C pneumoniae* serological assays and promote uniformity in laboratory practice. [Kalayoglu et al, 2002] [Ngeh and Gupta, 2004]

Newer serological methods such as the enzyme-linked immunosorbent assays (ELISA) have been developed and readily available as commercial kits. [Ngeh and Gupta, 2004] [Ngeh et al, 2004(a)] These ELISAs give qualitative results that can be read by photometric machine rapidly and objectively. [Ngeh et al, 2004(a)] [Ieven and



Hoymans, 2005] Because ELISAs can afford a relatively high throughput and objectivity, they are increasingly used in seroepidemiological studies. [Ngeh and Gupta, 2004] [Ngeh et al, 2004(a)] Preliminary data suggest that ELISA has a good sensitivity, specificity, and reproducibility when compared to MIF. [Ngeh and Gupta, 2004] [Ngeh et al, 2004(a)] However, like the MIF, ELISA is still far from perfect as a diagnostic tool, and will require further standardisation and improvement. [Ngeh and Gupta, 2004] [Ngeh et al, 2004(a)]

Serological markers such as immunoglobulin titres tend to fluctuate over time, and there is variability both within individuals and across populations. [Kalayoglu et al, 2002] [Leinonen and Saikku, 2002] [Ngeh and Gupta, 2004] Moreover, *C pneumoniae* infection is more common in autumn or winter seasons, and during cyclical epidemics. [Dugan et al, 2002] [Ngeh and Gupta, 2004] The time of sampling will therefore affect the results of serological studies. In older age groups, the seroprevalence of *C pneumoniae* is over 70%, making comparison of the actual, though small, difference in seropositivity between the cases and the controls difficult. [Leinonen and Saikku, 2002] [Ngeh, 2000] There were also reports suggesting a poor correlation between endovascular or intracellular *C pneumoniae* infection or detection (e.g. by culture / polymerase chain reaction) and serological positivity. [Leinonen and Saikku, 2002] [Ngeh et al, 2002] [Apfalter, 2006]

Nevertheless, obtaining patients' serum and other blood components remain a relatively convenient and non-invasive method to study for *C pneumoniae* infection. Currently, serological methods alone may not reliably detect chronic or persistent *C pneumoniae* infection in a subgroup of patients that might benefit from antibiotic

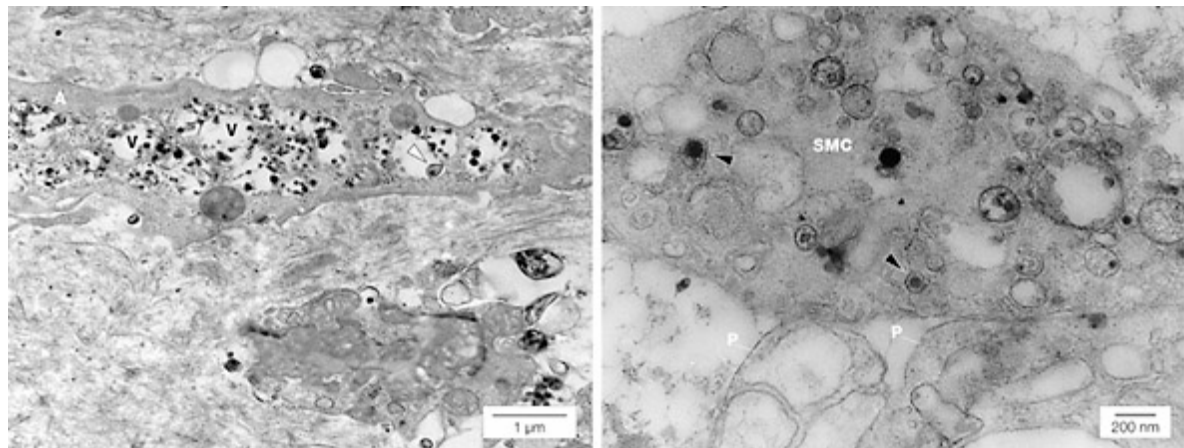
interventional therapy. [Ngeh and Gupta, 2004] It remains unclear which classes and titres of antibodies in fact represent acute first or reinfection, past, persistent but inactive, or chronically active *C pneumoniae* infection. [Apfalter, 2006] *C pneumoniae* serology has also been criticized as problematic in terms of specificity, reproducibility, and uncertainty regarding the clinical meaning of specific titres. [Apfalter, 2006] Indeed, investigators are studying other blood markers such as *C pneumoniae* immune complexes and circulating leucocytes e.g. macrophages with detectable *C pneumoniae* DNA and mRNA as markers of underlying infection. [Kalayoglu et al, 2002] [Ngeh and Gupta, 2004] Seroepidemiological studies have progressed to examine for other blood markers of infections implicated in atherosclerosis, to correlate inflammatory markers such as C-reactive protein (CRP) in conjunction with *C pneumoniae* serology, and have shown association between ‘infectious’ and ‘inflammatory’ burdens and atherosclerosis. [Leinonen and Saikku, 2002] [Ngeh and Gupta, 2004]

#### **1.7.3.2. Evidence linking *Chlamydia pneumoniae* and atherosclerosis: Pathological specimen findings**

Shor and colleagues provided the first evidence that *C pneumoniae* can be detected within atherosclerotic or CHD specimen and visualised with electronmicroscopy (EM) in 1992 (see Figure 2). [Shor and Phillips, 2000] [Ngeh and Gupta, 2004] Since then, many (probably over 60) published reports have demonstrated the presence of *C pneumoniae* not only in coronary arteries, but also in cerebral, carotid, internal mammary, pulmonary, aorta, renal, iliac, femoral and popliteal arteries, and occluded bypass grafts, obtained from postmortem and surgical atheromatous tissues.

[Leinonen and Saikku, 2002] [Rassu et al, 2001] [Ngeh and Gupta, 2004] Different techniques such as immunocytochemistry (ICC), polymerase chain reaction (PCR), *in situ* hybridisation (ISH), and EM were used in these studies. [Ngeh and Gupta, 2004] The rate of detection was about 50% (range varies from 0-100%) in atheromatous tissues, [Grayston, 2000] [Taylor-Robinson, 1998] but less than 1% in 'healthy' arteries free from obvious atheroma. [Ngeh and Gupta, 2004] Specifically, PCR evidence of *C pneumoniae* can be found in the middle cerebral artery [Virok et al, 2001] and other cerebral blood vessels [Vink et al, 2001]. *C pneumoniae* can also be detected in about 10% of non-cardiovascular and granulomatous tissues, showing a non-specific, ubiquitous distribution in human body. [Jackson et al, 1997] [Ngeh and Gupta, 2004] However, the distribution of *C pneumoniae* is known to correlate well with the distribution of atherosclerosis within the same individual. [Vink et al, 2001] [Ngeh and Gupta, 2004]

Figure 2. Transmission electron micrograph of smooth muscle cells in an early atherosclerotic lesion of the aorta positive for *Chlamydia pneumoniae* by polymerase chain reaction



On the left, a smooth muscle cell containing vacuoles (V) and *Chlamydia pneumoniae* elementary bodies (arrowhead) are demonstrated. The other fragmenting cell and actin (A) filaments are also shown. On the right, macrophage pseudopodia (P) are shown in contact with a fragment of smooth muscle cell (SMC) containing *Chlamydia pneumoniae* (arrowheads).

(Figure reproduced from: Shor A, Phillips J. Histological and ultrastructural findings suggesting an initiating role for *Chlamydia pneumoniae* in the pathogenesis of atherosclerosis: a study of fifty cases. Cardiovas J S Afr 2000;11:16-23, with permission. [Ngeh and Gupta, 2004])

The presence of *C pneumoniae* in atherosclerotic lesions is not unique. It is known that other micro-organisms such as *H pylori*, *CMV*, *Herpes simplex virus*, periodontal pathogens, *Mycoplasma pneumoniae* [Higuchi et al, 2000], have also been detected in human atherosclerotic tissues. [Fong, 2002] [Leinonen and Saikku, 2002] However, *C pneumoniae* is to date, the only micro-organism that can be cultured alive, albeit with difficulty, from atheromatous plaques [Leinonen and Saikku, 2002] (Table 4) [Kalayoglu et al, 2002] [Johnston et al, 2001] [Ngeh and Gupta, 2004].

Table 2. Pathological studies that have detected or cultured viable *C pneumoniae* from human atherosclerotic tissues

Year	Author	Specimen	Detection Method	Positive (%)	Viable (%)
1996	Ramirez	Coronary	Culture/EM/ICC/ISH/PCR	7/10 patients (70%)	1/10 patients (10%), by culture
1997	Jackson et al	Carotid	Culture/EM/ICC/ISH/PCR	12/16 specimens (75%), other than by culture	1/25 specimens (4%), by culture
1998	Maass et al	Coronary, CABG	Culture/PCR	21/70 patients (30%)	11/70 patients (16%), by culture
1999	Bartels et al	Saphenous vein CABG	Culture/PCR	8/32 specimens (25%)	5/32 specimens (16%), by culture
1999	Esposito et al	Carotid	PCR/RT-PCR	18/30 patients (60%)	10/30 patients (33%), presence of chlamydial mRNA by RT-PCR
2000	Karlsson et al	AAA	Culture/IHC	21/26 patients (81%)	10/25 patients (40%), by culture
2000	Apfalter et al	Carotid, coronary, AAA, iliac, femoral, AV	Culture/DIF/PCR	-	3/38 specimens (7.9%)
2001	Johnston et al	Carotid	PCR/RT-PCR	19/48 specimens (40%)	18/48 specimens (38%), presence of chlamydial mRNA by RT-PCR

AAA= Abdominal aortic aneurysm, AV= Aortic valve, CABG= Coronary artery bypass graft, DIF= Direct, genus specific immunofluorescence staining, EM= Electron microscopy, ICC= Immunocytochemistry, IHC= Immunohistochemistry, ISH= *In situ* hybridisation, PCR= Polymerase chain reaction, RT-PCR= Reverse transcriptase-PCR

The methods used in histopathological studies i.e. EM, ICC, PCR, ISH, do not correlate with one another consistently, making comparison of studies in this area and others difficult. [Kalayoglu et al, 2002] [Leinonen and Saikku, 2002] [Ngeh and Gupta, 2004] PCR technique alone tends to give the lowest rate of *C pneumoniae* detection, whereas the highest rate of detection is by ICC or a combination of methods. [Kalayoglu et al, 2002] Further efforts are therefore required to improve and standardise these methods. [Ngeh and Gupta, 2004]

In spite of methodological limitations, pathological examinations have shown that *C pneumoniae* has a tropism for arterial tissues and atherosclerotic lesions. [Jackson et al, 1997] [Ngeh and Gupta, 2004] Although the mere presence of *C pneumoniae* or its antigens in atheroma do not prove a causal role, pathological studies nevertheless support the rejection of the notion that *C pneumoniae* is simply an innocent ‘bystander’. [Jackson et al, 1997] [Ngeh and Gupta, 2004] Increasingly, histopathological methods are used in conjunction with animal, immunological and antibiotic studies to examine for causal, mechanistic and treatment susceptibility effects of *C pneumoniae* in atherosclerosis. [Ngeh and Gupta, 2004]

#### **1.7.3.3. Evidence linking *Chlamydia pneumoniae* and atherosclerosis: Animal experimental models**

Historical precedents of animal models used to demonstrate infection-induced atherosclerosis include Benson’s *Streptococcus*-rabbit and Fabricant’s *Herpesvirus*-chicken experimental models in 1931 and 1978 respectively. [Leinonen and Saikku,

2002] [Ngeh and Gupta, 2004] In 1997, Fong, Laitinen and co-workers were amongst the first to report that repeated *C pneumoniae* infection through the respiratory tract could induce not only pneumonia, but also inflammatory and atherosclerotic changes in the aortas of rabbits. [Fong et al, 1997] [Laitinen et al, 1997] [Ngeh and Gupta, 2004] *Mycoplasma pneumoniae*, another atypical respiratory pathogen used as a control in the study, was not found to induce any atherosclerotic lesions. [Leinonen and Saikku, 2002] Further animal experimentation demonstrated that treatment using azithromycin, a macrolide antibiotic active against *C pneumoniae*, could - though not invariably - counteract or reduce the atherogenic effect of *C pneumoniae* in the aortas of infected animals. [Dugan et al, 2002] [Leinonen and Saikku, 2002] [Ngeh and Gupta, 2004]

Animal models are invaluable laboratory tools used to study the synergistic effects of some of the well-established cardiovascular risk factors e.g. atherogenic diet and genetic predisposition, in the context of *C pneumoniae* induced atherosclerosis. [Ngeh and Gupta, 2004] Dietary supplementations with cholesterol in *C pneumoniae* infected rabbits have been shown to increase the animals' vascular intimal wall thickening. [Kalayoglu et al, 2002] [Fong, 2002] [Leinonen and Saikku, 2002] [Ngeh and Gupta, 2004] Genetically modified mouse such as ApoE-deficient mouse are known to develop atherosclerosis in the absence of a fatty diet. [Kalayoglu et al, 2002] [Fong, 2002] [Leinonen and Saikku, 2002] [Ngeh and Gupta, 2004] Repeated *C pneumoniae* infection has been shown to accelerate atherosclerotic lesion progression in this model, although a short 2-week course of azithromycin treatment did not reduce the size of the lesion. [Ngeh and Gupta, 2004] [Rothstein et al, 2001] Specifically, *C pneumoniae* infection was shown to increase T-lymphocyte influx in



the atherosclerotic plaques and to accelerate the formation of more advanced atherosclerotic lesions in ApoE3-Leiden mice. [Ezzahiri et al, 2002] [Ngeh and Gupta, 2004]

Further evidence suggests that the atherogenic effects of *C pneumoniae* infection were dependent on cholesterol and species specific to *C pneumoniae* as opposed to *C trachomatis*, in LDL-receptor-knockout transgenic mouse model. [Hu et al, 1999] [Ngeh and Gupta, 2004] On the other hand, the C57BL/6J mouse that does not develop atherosclerosis when fed a normal diet was shown to produce intimal thickening and carditis on repeated intranasal inoculation with *C pneumoniae*. [Campbell et al 1998] [Campbell et al, 1999] In addition, when fed a high fat diet, the infected C57BL/6J mice developed significantly larger atherosclerotic lesion areas compared with control mice. [Blessing et al, 2001] [Ngeh and Gupta, 2004]

More recently, larger mammals such as dogs and pigs were used in animal studies. [Sako et al, 2002] [Liuba et al, 2002] [Pislaru et al, 2003] [Ngeh and Gupta, 2004] Using EM, ICC and PCR methods, a Japanese group of researchers have reported the presence of viable *C pneumoniae* in canine atheromatous tissues. [Sako et al, 2002] Report from Sweden has demonstrated that acute *C pneumoniae* infection of pigs through the respiratory tract could cause profound endothelial dysfunction in coronary arteries, thus supporting the role of *C pneumoniae* in atherogenesis. [Liuba et al, 2002] More recently, a report using a pig model has shown that intracoronary and intrapulmonary *C pneumoniae* inoculation could induce coronary intimal proliferation in the absence of a lipid-rich diet. [Pislaru et al, 2003] However, direct administration

of macrophages infected with *C pneumoniae* into the pigs' coronary arterial wall was not associated with the development of coronary lesions. [Pislaru et al, 2003]

Animal models are useful to demonstrate a causal and temporal effect of *C pneumoniae* infection and subsequent development of atherosclerosis. [Ngeh and Gupta, 2004] They have shown that *C pneumoniae* infection could initiate and accelerate atherosclerotic process. [Kalayoglu et al, 2002] [Leinonen and Saikku, 2002] Moreover, the induced atherogenic process appears to correlate with increasing *C pneumoniae* infective dose and time from exposure, and accelerate with other atherogenic risk factors. [Leinonen and Saikku, 2002] [Ngeh and Gupta, 2004] The models could also help to define the role of antibiotic treatment in atherosclerosis. [Kalayoglu et al, 2002] [Ngeh and Gupta, 2004] However, exactly to what extent could laboratory induced atherogenesis in animal models reflects human atherosclerosis (which may take years to develop) remains an important issue to be resolved. [Ngeh and Gupta, 2004]

#### **1.7.3.4. Evidence linking *Chlamydia pneumoniae* and atherosclerosis: Molecular and immunological mechanisms**

Studies in this area have focused on the mechanistic roles of *C pneumoniae* in the inflammatory processes of atherosclerosis. [Ngeh and Gupta, 2004] A variety of studies have examined the interactions between *C pneumoniae* infections or antigens and components of athero-thrombotic tissues: (1) cells such as leucocytes, platelets, monocytes, endothelium, smooth muscle cells (SMC) and fibroblasts; (2) extra-

cellular matrix and substrates such as collagens, elastin, gelatin and fibronectin. [Ngeh and Gupta, 2004] [Loftus et al, 2002]

A range of molecules expressed by these cells have been identified: [Ngeh and Gupta, 2004]

1. Cytokines and chemokines: interleukins (e.g. IL-1, IL-2, IL-6, IL-8), tumour necrosis factors (e.g. TNF- $\alpha$ ), gamma interferon, monocyte integrins, monocyte chemotactic protein-1 (MCP-1), colony stimulating factor, growth factors (e.g. platelet-derived growth factor or PDGF, basic fibroblast growth factor or bFGF), tissue factor.
2. Adhesion molecules: vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), E-selectin, P-selectin.

These inflammatory cells and mediators are known to participate in complicated immunological cross-talks between *C pneumoniae* antigens and atherogenic / atherothrombotic processes. [Ngeh and Gupta, 2004]

*Chlamydia pneumoniae* can infect and reproduce in human macrophages / monocytes, endothelial and smooth muscle cells. [Gaydos et al, 1996] These are the key cells involved in atherogenesis that have been shown to support the growth and proliferation of *C pneumoniae* in laboratory studies. [Ngeh and Gupta, 2004] From pathological, animal and clinical observations, it is postulated that *C pneumoniae* can infect macrophages in human respiratory tract and be carried by circulatory monocytes to invade arterial sites that are either developing or prone to develop

atherosclerosis. [Ngeh and Gupta, 2004] At the endothelial surfaces of these sites, *C pneumoniae* antigens could cause endothelial dysfunction and monocyte activation, and initiate or exacerbate a series of endovascular immunological reactions. [Gupta, 1999] [Kalayoglu et al, 2002] [Ngeh and Gupta, 2004] Postulated mechanisms by which *C pneumoniae* could mediate proatherogenic effects include induction of these peripheral monocytes to secrete proinflammatory cytokines, [Kaukoranta-Tolvanen et al, 1996] association with cytotoxic (CD8) T cells, [Nadareishvili et al, 2001] and production of immune complexes [Linnanmaki et al, 1993] [Wimmer et al, 1996]

Experimental data has shown that *C pneumoniae* A-03 (a coronary strain) could stimulate the production of MCP-1, IL-8, and ICAM-1 by human endothelial cells in vitro. [Molestina et al, 1998] Circulating leucocytes e.g. monocytes and T-lymphocytes, attracted by various mediators to the activated endothelial surface, could themselves be activated and express further inflammatory molecules (e.g. ILs and TNFs), which contribute to further cellular recruitment and chronic endothelial cell damage. [Ngeh and Gupta, 2004] Indeed, the ‘response-to-injury’ hypothesis refers to the fact that endothelial cell damage is the crucial initial stage in atherogenesis. [Ngeh and Gupta, 2004] Moreover, a number of traditional cardiovascular risk factors and infections are known to cause endothelial injury, dysfunction and activation. [Ngeh and Gupta, 2004]

Monocytes and macrophages are the key transporters of LDL-cholesterol from systemic circulation into subendothelial space. [Kalayoglu et al, 2002] [Ngeh and Gupta, 2004] Indeed, *C pneumoniae* LPS has been identified as the antigen that could enhance LDL-cholesterol uptake and down-regulate cholesterol efflux in monocytes

and macrophages. [Kalayoglu et al, 2002] [Leinonen and Saikku, 2002] [Ngeh and Gupta, 2004] Once the monocytes and leucocytes (T-lymphocytes and neutrophils) are anchored by adhesion molecules onto the dysfunctional endothelial surface, transendothelial penetration into the subendothelial space occurs. [Koenig, 1999] [Molestina et al, 1999] [Ngeh and Gupta, 2004] Many of these monocytes loaded with oxidised LDL-cholesterol then transform into tissue macrophages known as foam cells. [Kalayoglu et al, 2002] [Fong, 2002] [Leinonen and Saikku, 2002] [Ngeh and Gupta, 2004] Indeed, Kalayoglu and Byrne in 1998 demonstrated that *C pneumoniae* LPS could induce not only LDL-cholesterol oxidation but also macrophage transformation into foam cells, a key atherogenic process. [Kalayoglu and Byrne, 1998] [Kalayoglu et al, 2002] Furthermore, *C pneumoniae* heat shock protein-60 (cHSP60) was found to induce cellular oxidation of LDL-cholesterol. [Kalayoglu et al, 1999] [Ngeh and Gupta, 2004] The foam cells are known to participate in inflammatory and immunological reactions that lead to a fibroproliferative response from the arterial SMCs and the formation of atherosclerotic plaque. [Kalayoglu et al, 2002] [Ngeh and Gupta, 2004] Moreover, *C pneumoniae* infected human SMCs have been shown to stimulate the production of IL-6 and bFGF that contribute to atherosclerotic fibrous plaque formation. [Kalayoglu et al, 2002] [Ngeh and Gupta, 2004]

*Chlamydial* cHSP60 and human hHSP60 were frequently found together in human atherosclerotic plaques. [Kol et al, 1998] [Ngeh and Gupta, 2004] Because they share sequence homology, cHSP60 could cross-react with hHSP60, causing antibody-mediated cytotoxicity. [Ngeh and Gupta, 2004] In contrast, *C pneumoniae*-reactive T lymphocytes in human atherosclerotic plaques represent specific cell-mediated

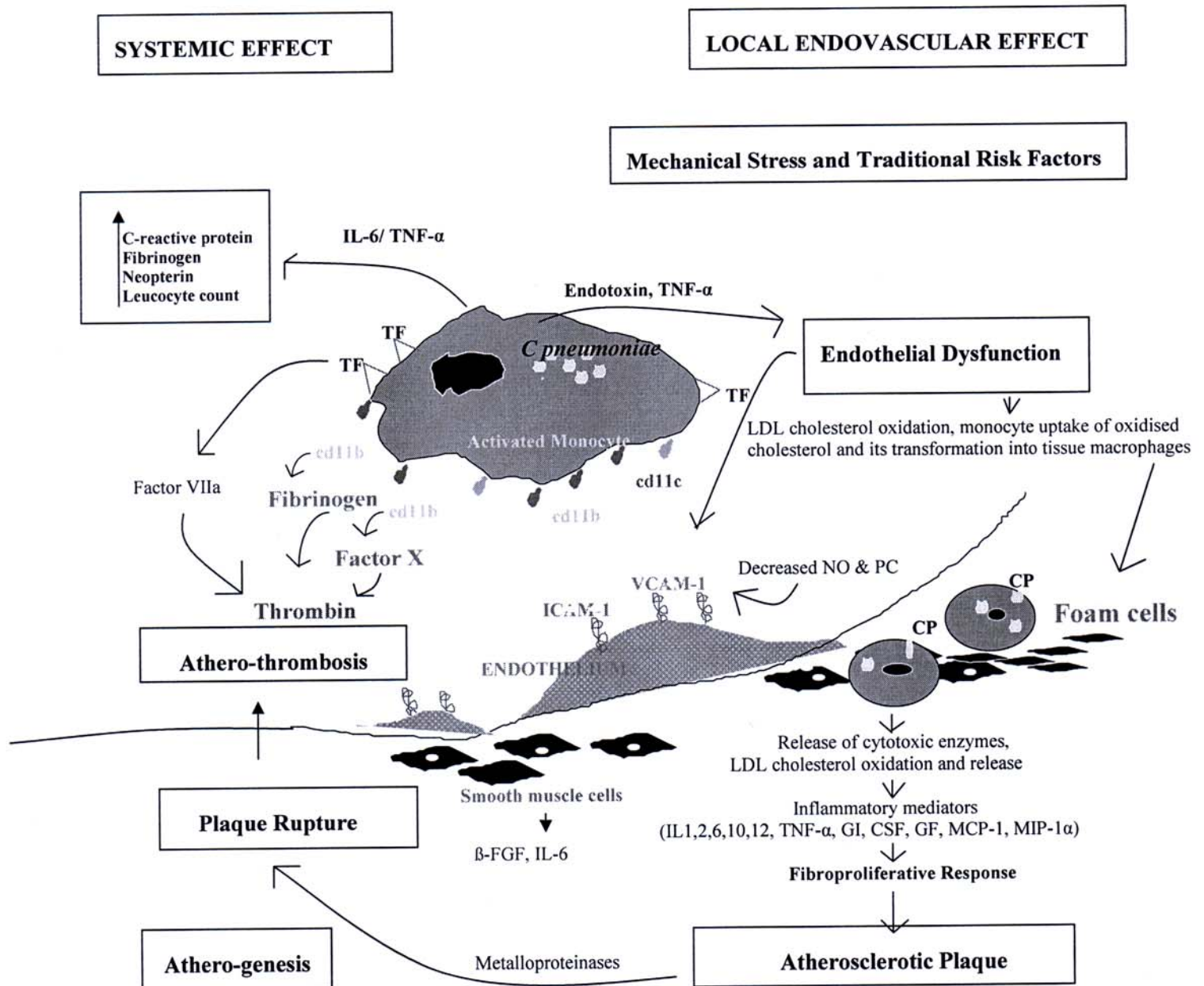
immunity. [Kalayoglu et al, 2002] [Leinonen and Saikku, 2002] [Ngeh and Gupta, 2004] These lymphocytes can produce gamma interferon and convert *C pneumoniae* into PB or persistent infection. [Kalayoglu et al, 2002] *C pneumoniae* can also inhibit apoptosis in infected host cells through inhibition of mitochondrial cytochrome-c release and caspase-3 activation, mediating a chronic infection. [Airenne et al, 2002] [Ngeh and Gupta, 2004]

HSP60 could activate macrophages to release TNF- $\alpha$  and matrix metalloproteinases that can degrade connective tissue and cause plaque rupture leading to acute atherothrombotic events. [Kalayoglu et al, 2002] [Fong, 2002] [Ngeh and Gupta, 2004] These adverse events are enhanced by an increased vascular tone resulting from decreased nitric oxide and prostacyclin production by dysfunctional endothelium, in a procoagulant environment due to tissue factor expression by activated monocytes. [Ngeh and Gupta, 2004] Apart from its local endovascular effects, *C pneumoniae* can also systemically interact with circulating blood cells; stimulate hepatic synthesis of inflammatory markers e.g. CRP, fibrinogen and neopterin; disturb lipoprotein metabolism; enhance clotting cascade; and promote atherothrombotic events (see Figure 3 below). [Ngeh and Gupta, 2004] [Gupta, 1999]

More recent studies have reported specific signalling pathways involved in *C pneumoniae* pathogenic mechanisms. [Ngeh and Gupta, 2004] For example, *C pneumoniae* and cHSP60 were shown to stimulate human vascular SMCs proliferation through the activation of toll-like receptor 4 that acts as antigenic sensor for *C pneumoniae*, with signalling through the p44/p42 mitogen-activated protein kinase (MAPK) pathway. [Sasu et al, 2001] The activation of nuclear transcription

factors such as NF- $\kappa$ B and Activator Protein-1 (proliferative intracellular signals) by *C pneumoniae* was found to induce vascular SMCs proliferation, an effect that can be abolished by the antibiotic azithromycin. [Miller et al, 2000] NF- $\kappa$ B activation in *C pneumoniae* infected cells was shown to induce the production of pro-inflammatory and pro-coagulant substances. [Dechend et al, 1999] [Molestina et al, 2000] [Miller et al, 2000] In addition, NF- $\kappa$ B was found to regulate many of the inflammatory vascular effects in hypercholesterolaemia. [Wilson et al, 2000] [Lindsberg and Grau, 2003]

In conjunction with traditional atherosclerotic risk factors, Figure 3 [Ngeh and Gupta, 2004] [Kalayoglu et al, 2002] [Gupta, 1999] in the following page showed that *C pneumoniae* infected monocyte may be activated and contribute towards endothelial dysfunction and subsequent foam cell transformation and inflammatory response in the subendothelial space. Monocyte activation and endothelial dysfunction also lead to secretion of cytokines and acute-phase proteins, expression of adhesion molecules, and up-regulation of tissue factor and monocyte integrins. These processes may either act independently or interact leading to atherogenesis and athrothrombosis.



**Figure 3. The roles of *C pneumoniae*, monocytes, and inflammatory markers in athero-genesis and athero-thrombosis: interplay between molecular and immunological mechanisms**

Cd11b/c= monocyte integrins, Cp= *Chlamydia pneumoniae*, CSF= colony stimulating factor, β-FGF= β-fibroblast growth factor, GF= growth factor, GI= gamma interferon, ICAM/VCAM= Intercellular/Vascular cell adhesion molecules, IL= interleukin, LDL= low density lipoprotein, MCP= monocyte chemoattractant protein, MIP= macrophage inflammatory protein, NO= nitric oxide, PG= prostacyclin, TF= tissue factor, TNF= tumour necrosis factor.



In keeping with traditional theories of atherogenesis, various mechanistic, molecular and immunological studies have demonstrated a pathogenic role of *C pneumoniae* infection in different stages of atherosclerosis. Further understanding and progress in this area of research – the ‘infectious hypothesis of atherosclerosis’ – may provide important ‘laboratory-based’ causal evidence of *C pneumoniae* or other micro-organisms in atherosclerosis; and stimulate ‘clinical’ prevention, interventional and therapeutic strategies. [Ngeh and Gupta, 2004]

#### **1.7.3.5. Evidence linking *Chlamydia pneumoniae* and atherosclerosis: Antibiotic treatment studies**

Most of the investigations in this area involved clinical antibiotic interventional trials in the secondary prevention of coronary heart disease (CHD). Others have examined the impact of antibiotic treatment on CHD in retrospective epidemiological studies. The results of these studies in the context of CHD are largely negative or at best, inconclusive. Apart from CHD, antibiotic treatment studies in the clinical context of abdominal aortic aneurysm (AAA), peripheral arterial disease (PAD), and carotid artery stenosis are emerging (see Table 5). [Ngeh and Gupta, 2004] [Higgins, 2003] [Dugan et al, 2002] [Grayston, 2003] [Cercek et al, 2003] [Hansen et al, 2001] [Melissano et al, 1999] [Parchure et al, 2002] [Hillis et al, 2004] [Grayston et al, 2005] [Cannon et al, 2005] [Vainas et al, 2005] [Jespersen et al, 2006]

**Table 5. Antibiotic treatment studies and atherosclerotic vascular diseases**

<b>Trial/Year</b>	<b>Population/Number</b>	<b>Antibiotic/Course</b>	<b>Trial duration</b>	<b>Results</b>
<b>Antibiotic Treatment and Coronary Heart Disease</b>				
St George's Hospital, London, UK. RCT, 1997	Post MI, 220 males	Azithromycin, 3 or 6 days	1.5 years	Positive
ROXIS, Argentina. RCT, 1997	ACS, 202 (76% men)	Roxithromycin, 30 days	6 months	Positive at 1 month, negative at 3 and 6 months
University of Washington, USA RCT, 1998	Post PTCA, 88	Azithromycin, 28 days	6 months	Positive
ACADEMIC, USA. RCT, 1999	Stable CAD, 302 (89% men)	Azithromycin, 3 months	2 years	Negative
ISAR-3, Germany. RCT, 2001	Post coronary stenting, 1010 (78% men)	Roxithromycin, 28 days	1 year	Negative, restenosis reduced in patients with titres 1/512
Siriraj Hospital, Bangkok, Thailand. RCT, 2001	ACS, 84 (63% men)	Roxithromycin, 30 days	90 days	Negative
STAMINA, UK. RCT, 2002	ACS, 325 (69% men)	Azithromycin or Amoxicillin, and Metronidazole, 7 days	1 year	Positive
WIZARD, International multicentre trial. RCT, 2002	Stable CAD, 7724 (83% men)	Azithromycin, 3 months	1-4 years (mean 2.5 years)	7% event reduction (hazard ratio 0.93, P= 0.23)
CLARIFY, Finland. RCT, 2002	ACS, 148 (70% men)	Clarithromycin, 3 months	138-924 days (mean 555 days or 1.5 years)	Positive
St George's Hospital, London, UK. RCT, 2002	Stable CAD, 40 males	Azithromycin, 3 days, then weekly for 4 weeks	5 weeks	Improvement in FMD of brachial artery, P< 0.005

AZACS, USA. RCT, 2003	ACS, 1439 (74% men)	Azithromycin, 5 days	6 months	Negative
ANTIBIO, Germany. RCT, 2003	ACS, 872 (79% men)	Roxithromycin, 6 weeks	1 year	Negative
Hillis et al, Edinburgh, UK RCT, 2004	ACS, 141	Azithromycin, 5 days	3 months	soluble intercellular adhesion molecule reduced
ACES, USA. RCT, 2005	Stable CAD, 4012	Azithromycin, weekly for 1 year	4 years	Negative
PROVE IT, International Multicentre trial. RCT, 2005	ACS, 4162	Gatifloxacin, 2 weeks, then 10-day course monthly for 2 years	2 years	Negative
Berg et al, Netherlands. RCT, 2005	Pre-CABG surgery, 473	Clarithromycin, mean= 16 days	2 years	Negative
CLARICOR, Denmark. Multicentre trial. RCT, 2006	Stable CAD 4373	Clarithromycin, 2 weeks	3 years	Negative

---

**Antibiotic Treatment and Carotid Atherosclerosis (CA), Abdominal Aortic Aneurysm (AAA), and Peripheral Arterial Disease (PAD)**

---

Italy RT, 1999	<i>C pneumoniae</i> burden in CA, 32 (16 males)	Roxithromycin, 17-35 days (mean 26 days)	17-35 days (mean 26 days)	Positive, P= 0.034
Finland RCT, 2001	AAA growth, 32 (29 males)	Doxycycline, 3 months	1.5 years	Positive, P= 0.03
Denmark RCT, 2001	AAA growth, 92 males	Roxithromycin, 28 days	1.5 years	Positive, P= 0.02
Switzerland RCT, 2002	PAD progression, 40 males	Roxithromycin, 28 days	2.7 years	Positive, P< 0.05
Germany RCT, 2002	CA progression, 272 (56% men)	Roxithromycin, 30 days	2 years	Positive, P< 0.01
SPACE Trial, Netherlands RCT, 2005	PAD, 509	Azithromycin, 3 days	2 years	Negative

---

ACS= Acute coronary syndrome, CAD= Coronary artery disease, FMD= Flow mediated dilation, MI= Myocardial infarction, PTCA= Percutaneous transluminal coronary angioplasty, RCT= Randomised controlled trial

In 1997, the positive results of two pilot antibiotic treatment studies were reported independently by Gupta and colleagues in the United Kingdom (UK) [Gupta et al, 1997] and Gurfinkel and colleagues in Argentina [Gurfinkel et al, 1997]. In the UK study, 60 male survivors of acute myocardial infarction (MI) with persistently raised *C pneumoniae* IgG antibody titres  $\geq 1:64$  were randomised to receive placebo or a macrolide antibiotic azithromycin (500mg per day for 3 or 6 days). Apart from a reduction in cardiovascular events at 18 months in the treatment group, there was also a reduction in several inflammatory / monocyte activation markers and *C pneumoniae* IgG antibody titres, in comparison with the placebo group and non-randomised group with high antibody titres.

The Argentinian ROXIS (Roxithromycin in Ischaemic Syndromes) study randomised hospitalised patients with unstable angina or non Q-wave MI (n= 202) to receive a placebo or another macrolide antibiotic roxithromycin (150mg twice daily) for 30 days. [Gurfinkel et al, 1997] At 1 month, a reduction in the combined triple end-point (severe recurrent angina, acute MI, ischaemic death) in the treatment group was reported. However, at 3 and 6 months, the beneficial effect of antibiotic treatment became non-significant, although inflammatory markers such as the CRP level decreased more significantly in the treatment group. This implied that a longer duration of antibiotic treatment trial might have been necessary to confer more lasting protective effects. [Ngeh and Gupta, 2004]

Another pilot antibiotic treatment study was reported in 1998 [Dugan et al, 2002]. Eighty-eight patients who had had percutaneous transluminal coronary angioplasty were randomised to receive azithromycin 500mg daily or placebo for 2 days, followed by azithromycin 250mg daily or placebo for 28 days. The investigators found that patients in the treatment group had less re-stenosis (9%) than those in the placebo group (16%), and also less recurrent angina (40% versus 60%, respectively). However, the positive results were not sustained in a larger follow-up study.

In 1999, the findings of Azithromycin in Coronary Artery Disease Elimination of Myocardial Infection with Chlamydia (ACADEMIC) study were reported from the United States. [Anderson et al, 1999] The ACADEMIC study randomised 302 subjects with elevated *C pneumoniae* antibody titres and CHD to receive a placebo or a 3-month course of azithromycin (500mg/day for 3 days, then 500mg/week). The investigators found no difference in clinical outcome at 6 months and at 2 years in both placebo and treatment groups. [Anderson et al, 1999] [Muhlestein et al, 2000] However, a reduction in inflammatory markers such as CRP, IL-1, IL-6, and TNF- $\alpha$  was found at 6 months. [Anderson et al, 1999]

The ISAR-3 (Intracoronary Stenting and Antibiotic Regime trial-3) study published in 2001 investigated whether roxithromycin could prevent *C pneumoniae*-associated re-stenosis after coronary stent placement. [Neumann et al, 2001] Consecutive patients were randomised in a double-blind fashion to receive a placebo (n= 504) or 300mg daily of roxithromycin (n= 506) for 28 days. The investigators reported no real difference in outcome as the rate of angiographic stenosis was 31% in the treatment group versus 29% in placebo group at follow-up. The combined 1-year mortality and

MI rates were also similar in both groups i.e. 7% in treatment group versus 6% in placebo group. However, in those patients with high *C pneumoniae* antibody titres i.e. 1:152, the investigators found that roxithromycin treatment significantly reduced the rate of re-stenosis after coronary stenting. This suggested that selective usage of roxithromycin in some patients with *C pneumoniae* infection might reduce post coronary stenting re-stenosis. [Ngeh and Gupta, 2004]

In a small Thai study, 84 patients with acute coronary syndrome were randomized to receive roxithromycin (150mg BD) or placebo for 30 days. [Leowattana et al, 2001] [Higgins, 2003] [Grayston, 2003] After a follow-up period of 90 days, the investigators found no significant difference in adverse cardiovascular events between the roxithromycin and placebo groups (17 versus 16).

Several antibiotic treatment studies were reported in 2002. The South Thames Trial of Antibiotics in Myocardial Infarction and Unstable Angina (STAMINA) is another small UK study. [Higgins, 2003] [Grayston, 2003] In this study, 325 patients with acute coronary syndromes (unstable angina or first MI) were randomised to receive a 1-week course of either placebo; amoxicillin (500mg BD), metonidazole (400mg BD), and omeprazole (20mg BD); or azithromycin (500mg OD), metronidazole (400mg BD), and omeprazole (20mg BD). The investigators found that antibiotic treatment significantly reduced adverse cardiac events at 12 months. This favourable treatment effect appeared to be independent of seropositivity to *C pneumoniae* and *H pylori*, and there was no difference found between the azithromycin and amoxycillin groups. The CRP and fibrinogen levels were found to decrease in the antibiotic treatment groups.

The WIZARD (Weekly Intervention with Zithromax for Atherosclerosis and Its Related Disorders) study is a large randomised, placebo controlled study that has enrolled more than 7,000 patients who had an MI at least 6 weeks before enrolment and had *C pneumoniae* IgG titres > 1:16. [O'Connor et al, 2003] Of these, 3,868 patients were randomised to receive azithromycin 600mg/day for 3 days, then 600mg weekly for 11 weeks, and 3,856 patients received placebo. At the 2-year follow-up, the investigators found a 7% reduction in the composite primary end point of all-cause mortality, recurrent MI, hospitalisation, and revascularisation (hazard ratio= 0.93, P= NS).

The Clarithromycin in Acute Coronary Syndrome Patients in Finland (CLARIFY) study reported favourable results with antibiotic treatment. [Sinisalo et al, 2002] In this small study, 148 patients with unstable angina or acute non-Q-wave coronary events were randomised to receive either clarithromycin 500mg or a placebo once daily for 85 days. During the initial 3-month treatment period, there were 11 patients in the treatment group compared to 19 patients in the placebo group that met the combined primary end point of death, MI, or unstable angina (P= 0.1). Over an average period of 555 days, there were also fewer patients experiencing cardiovascular events in the treatment group (16 versus 27 in the placebo group; P= 0.03).

In the AZACS (Azithromycin in Acute Coronary Syndromes) study, 1439 patients with unstable angina or acute MI were randomly assigned to receive azithromycin

(500mg for 1 day, then 250mg for 4 more days) or placebo. [Cercek et al, 2003] Patients were followed-up for 6 months. There were no differences found between the two groups in terms of adverse outcome such as the incidence of death, recurrent MI and revascularisation.

The ANTIBIO was a German study reported in 2003. [Zahn et al, 2003] A total of 872 patients with an acute MI were randomized to receive either roxithromycin 300mg or placebo daily for 6 weeks. After a 12-month follow-up period, the investigators found that roxithromycin treatment did not reduce death and adverse cardiac events when compared with the placebo group.

The ACES (Azithromycin and Coronary Events Study) was an American study published in 2005. [Grayston et al, 2005] In this study, 4,012 patients with stable coronary artery disease were randomised to receive either 600mg of azithromycin or placebo weekly for one year. The mean duration of follow-up was 3.9 years. The results showed that there was no significant risk reduction in the azithromycin group as compared with the placebo group with regard to cardiac events, death from any cause, or stroke.

The PROVE-IT (Pravastatin or Atorvastatin Evaluation and Infection Therapy) study was a double-blind, randomised, placebo-controlled trial published in 2005. [Cannon et al, 2005] In this study, 4162 patients who had been hospitalised for an acute coronary syndrome within the preceding 10 days were randomized to receive 400 mg of gatifloxacin daily during an initial 2-week course of treatment that began 2 week



after randomisation, followed by a 10-day course every month for a mean duration of 2 years, or placebo. The results showed that long term treatment with gatifloxacin, a bactericidal antibiotic effective against *C pneumoniae*, did not reduce the rate of cardiovascular events, death from all causes, or stroke.

In 2005, another study reported that treatment with clarithromycin (mean duration= 16 days) prior to coronary artery bypass graft surgery did not prevent subsequent cardiac events or mortality during a 2-year follow-up period. [Berg et al, 2005(a)] The investigators also reported that *C pneumoniae* DNA was not present in vascular tissue, including atherosclerotic plaques of patients with advanced coronary artery disease. [Berg et al, 2005(b)] They concluded that antibiotic treatment was unlikely to have any effect in patients with advanced atherosclerosis.

The CLARICOR trial published in 2006 was a randomised placebo controlled multicentre trial that assessed a 2-week treatment effect of clarithromycin (500 mg per day) in patients with stable coronary heart disease. [Jespersen et al, 2006] 2,172 participants were randomised to clarithromycin and 2,201 to placebo. The study found no significant beneficial effects of clarithromycin on adverse cardiovascular events. In addition, the study found that cardiovascular mortality was significantly higher in patients treated with clarithromycin (hazard ratio= 1.45; 95% CI= 1.09 to 1.92; P= 0.01). The long term safety issue of clarithromycin treatment in patients with stable ischaemic heart disease was raised. [Jespersen et al, 2006]

Contradictory results have been reported in several large-scale retrospective, antibiotic treatment case-control studies. In the first study involving 3,315 cases and 13,139 controls, Meier and colleagues provided indirect evidence for a positive association between past usage of antibiotics such as tetracycline or quinolones (but not macrolides) and the reduction in risk of first-time MI. [Meier et al, 1999] However, the results of another similar study (1,796 cases vs 4,882 controls) suggested that past usage of tetracycline, doxycycline or erythromycin (a macrolide) was not associated with the risk of first MI. [Jackson et al, 2000] Further study on the usage of a variety of antibiotics and their relationship to first MI in a population involving 354,258 patients was also reported with negative results. [Luchsinger et al, 2002] Another study involving a database of 26,195 patients concluded that exposure to anti-chlamydial antibiotics (tetracyclines, macrolides and quinolones) during the 3 months after an acute MI was associated with a small survival benefit, whereas exposure during the 6 months before an acute MI did not affect survival. [Pilote et al, 2002] In another case-control study nested within a cohort of 29,937 elderly subjects on antihypertensive treatment, a trend was observed for a decreased risk of acute MI in patients receiving a prescription for antichlamydial antibiotics in the preceding 3 months (Odds ratio= 0.68; 95% CI= 0.46 to 1.00); but antibiotics without antichlamydial activity showed no benefit in reducing the risk of MI. [Brassard et al, 2003(a)] Using the same study design and cohort of patients, the investigators reported no clear, consistent associations between overall antibiotic use and stroke; although penicillin was the only individual antibiotic class that showed a protective association between penicillin use and stroke. [Brassard et al, 2003(b)] Indeed, an earlier study has concluded that exposures to short courses of antibiotics were not

associated with lower risk of ischaemic stroke in patients aged 65 years and older [Luchsinger et al, 2001]

Several meta-analyses on randomized controlled trials (enrolling almost 20,000 patients) that evaluated the effect of antibiotics on the secondary prevention of coronary artery diseases have been reported. [Etminan et al, 2004] [Wells et al, 2004] [Andr ws et al, 2005] [Baker and Couch, 2007]. These meta-analyses concluded that evidence available to date did not demonstrate an overall benefit of antibiotic or macrolide therapy in the reduction of cardiovascular events or mortality in patients with coronary artery disease.

A positive treatment effect of roxithromycin on small abdominal aortic aneurysm (AAA) expansion rate especially in patients with *C pneumoniae* IgA seropositivity was reported in 2001. [Vammen et al, 2001] The investigators in this study randomised 92 male subjects to receive either roxithromycin 300mg per day or placebo for 28 days, and patients were followed up for a mean period of 1.5 years. They found that the expansion rate of AAA was reduced by 44% i.e. 1.56mm per year in the treatment group versus 2.80 mm per year in the placebo group ( $p= 0.02$ ). During the second year, the difference was only 5%. The results remained significant even after multiple linear and logistic regression statistical analyses. A similar positive result of a significant reduction in the AAA expansion rate was reported in another randomised, double-blind, placebo-controlled pilot study (32 patients followed up for 18 months) using the antibiotic doxycycline. [Mosorin et al, 2001]

In the clinical context of peripheral arterial disease (PAD), a pilot study that randomised 40 *C pneumoniae* seropositive men suffering from peripheral arterial occlusive disease to receive either roxithromycin 300mg daily or placebo for 28 days reported its results in 2002. [Wiesli et al, 2002] During a follow-up period of 2.7 years, the investigators found that there were significantly fewer numbers of invasive revascularisation per patient in the treatment group (5 interventions performed on 4 patients) in comparison with the placebo group (29 interventions on 9 patients), even after multiple regression analyses. They also reported that limitation of walking distance to 200 metres or less occurred in fewer patients in the treatment group than in the placebo group (4 vs 13). Interestingly, they also observed a significant regression of the size of soft carotid plaques in the roxithromycin treated patients. [Wiesli et al, 2002] However, a subsequent randomised, placebo-controlled study involving 509 patients with PAD reported that a 3-day course of azithromycin (500mg daily) did not show any benefit for survival or ankle pressure during 2 years of follow-up. [Vainas et al, 2005]

In 1999, an Italian randomised study reported that treatment with a course of roxithromycin (150mg twice daily for a mean of 26 days) before carotid endarterectomy appeared effective in reducing the bacterial burden of *C pneumoniae* within carotid atherosclerotic specimens using PCR detection method. [Melissano et al, 1999] In 2002, a group of investigators in Germany reported that roxithromycin treatment reduced progression of early carotid atherosclerosis in *C pneumoniae* seropositive patients with ischaemic stroke. [Sander et al, 2002] They randomized 272 patients with ischaemic stroke aged over 55 years to receive either roxithromycin 150 mg twice daily or placebo for 30 days. In the 62 *C pneumoniae* seropositive patients

that received roxithromycin, there was a significantly decreased common carotid artery intima-to-media thickness (IMT) progression after 2 years, when compared with *C pneumoniae* seropositive patients (n= 63) that received placebo. They observed no significant difference in IMT progression in *C pneumoniae* seronegative patients that received either roxithromycin (n= 74) or placebo (n= 73). In addition, no significant difference in the occurrence of future cardiovascular events (stroke, MI or vascular death) was observed in either the treatment or placebo groups. The CRP levels decreased after roxithromycin treatment more significantly in the *C pneumoniae*- seropositive than in the seronegative patients, when compared with their respective pre-treatment values. When the CRP levels in these 2 treatment groups were compared with their respective placebo groups, a significant difference was only observed in the *C pneumoniae* positive group. These results implied that roxithromycin might have a greater anti-chlamydial than its anti-inflammatory effects. [Grayston et al, 2003] [Ngeh and Gupta, 2004] However, these investigators subsequently reported that the IMT progression increased again during the third and fourth year to similar values as before treatment, and suggested that antibiotic therapy had limited positive impact on early atherosclerosis progression in *C pneumoniae* positive patients. [Sander et al, 2004] A need for long-term or intermittent treatment may be indicated, since *C pneumoniae* may persist in monocytes in a latent state. [Elkind and Cole, 2006]

Rather than focussing on adverse clinical events as outcome measures, a recent UK study assessed the treatment effect of azithromycin on endothelial function in patients with CHD that were seropositive to *C pneumoniae*. [Parchure et al, 2002] The investigators randomised 40 male patients with CHD and *C pneumoniae* IgG antibody

to receive either azithromycin (500mg per day for 3 days, then 500mg once weekly for 4 weeks) or placebo. They found that patients in the treatment group, irrespective of their initial *C pneumoniae* antibody titres, had a significant improvement in flow-mediated dilation (FMD) of the brachial artery when compared to those in the placebo group. Azithromycin treatment also resulted in a significant decrease in E-selectin and von Willebrand factor (both markers of endothelial dysfunction) but not CRP levels. They concluded that azithromycin treatment had a favourable effect on endothelial function in patients with CHD and evidence of *C pneumoniae* infection. Indeed, another randomised treatment study reported that after an acute coronary syndrome, a 5-day course of azithromycin reduced levels of soluble intercellular adhesion molecule-1, a marker of endothelial cell activation. [Hillis et al, 2004]

Several important issues need to be addressed in future antibiotic treatment studies. The macrolides and tetracyclines used in the treatment studies are known to possess anti-inflammatory and immuno-modulatory effects, in addition to their anti-chlamydial activities. [Higgins, 2003] [Leinonen and Saikku, 2002] [Ngeh and Gupta, 2004] The relative contribution of these independent pharmacological properties in treatment studies and their concurrent anti-microbial effects on other micro-organisms need to be delineated. [Ngeh and Gupta, 2004] Furthermore, the optimal dose and dosing regime, duration and even cycles of antibiotic treatment in future studies will need to be tested; since reinfection with *C pneumoniae* is common. [Andraws et al, 2005] Previous study has found that *C pneumoniae* infection in circulating human monocytes was not ameliorated by antibiotics. [Gieffers et al, 2001] While under immune stress, *C pneumoniae* may transform into PB and becomes resistant to treatment yet capable of inciting inflammation. (Figure 1) [Kalayoglu et al, 2002]

[Andraws et al, 2005] Apart from its bacteriostatic or bacteriocidal property, whether an antimicrobial can reliably eradicate different forms of *C pneumoniae* (including PB) or can penetrate atheromas effectively, will also need to be explored and determined.

[Ngeh and Gupta, 2004] [Andraws et al, 2005]

It is also important to develop reliable methods that can detect true markers reflecting chronic / persitent *C pneumoniae* infection or high infectious burden in individuals targeted in future antibiotic treatment studies. [Ngeh and Gupta, 2004] As *C pneumoniae* is known to associate with different stages of atherosclerosis, it will be important to target homogenous populations with similar atherosclerotic burden and in different age groups to verify treatment effects. [Ngeh and Gupta, 2004] Hence, antibiotic treatment studies in the setting of primary prevention in high risk population with silent / subclinical atherosclerosis should be considered. [Ngeh and Gupta, 2004] Future antibiotic treatment studies should increasingly utilise and incorporate other surrogate (e.g. radiological, pathological, blood) markers of plaque activities (which may be clinically silent) to assess treatment effects, rather than using clinical vascular events as the sole outcome measure. [Ngeh and Gupta, 2004]

#### **1.7.4. *Chlamydia pneumoniae*, atherosclerotic risk factors and Koch's postulates**

Because atherosclerosis is a complicated multifactorial disease with many clinical variables, it is important to assess the role of *C pneumoniae* in the context of other well established atherosclerotic risk factors. [Ngeh and Gupta, 2004] Interestingly, *C pneumoniae* infection is known to be associated with classical vascular risk factors

such as smoking and those of the metabolic syndrome: hypertension, dyslipidaemia, diabetes, obesity. [Kalayoglu et al, 2002] [Fong, 2002] [Leinonen and Saikku, 2002] [Ngeh and Gupta, 2004] In addition, *C pneumoniae* infection is also more commonly associated with other non-modifiable vascular risk factors such as male sex, age, and certain genetic predisposition (HLA DRII genotype 13a or 17) [Dahlen et al, 1995]. [Leinonen and Saikku, 2002] [Ngeh and Gupta, 2004] Furthermore, *C pneumoniae* infection is also associated with novel vascular risk factors such as hyperhomocysteinaemia [Stanger et al, 2002] and elevated CRP [Kalayoglu et al, 2002] [Leinonen and Saikku, 2002] [Ngeh and Gupta, 2004]. As all major vascular risk factors are known to correlate with one another in a synergistic way, it is conceivable that chronic *C pneumoniae* infection may represent a forceful inflammatory stimulus that will interact with all these major vascular risk factors in the pathogenesis and clinical manifestations of atherosclerosis. [Ngeh and Gupta, 2004] Indeed, studies that examined specific mechanistic interactions (e.g. iron and lipid metabolisms, immunological expressions) between *C pneumoniae* and specific vascular risk factors (e.g. gender, dyslipidaemia, genetic polymorphisms) are emerging. [Leinonen and Saikku, 2002]

*C pneumoniae* is unlikely to be the sole cause of atherosclerosis, a complicated disease with its many clinical manifestations. [Ngeh and Gupta, 2004] This is in contrast to the *H Pylori*-peptic ulcer disease causal link, where the latter is a more straightforward disease with fewer risk factors and clinical variables. Traditionalists may demand a strict fulfilment of Koch's postulates in order to establish that *C pneumoniae* infection is a cause of atherosclerosis. [Ngeh and Gupta, 2004] Although this is not always possible in the case of *C pneumoniae*-atherosclerosis association, it



must be remembered that the accepted H pylori-peptic ulcer causal link does not always satisfy Koch's postulates either. [Ngeh and Gupta, 2004] (see Table 6) [Ngeh and Gupta, 2000]

**Table 6. Koch's postulates for infectious diseases: *Chlamydia pneumoniae* in atherosclerosis versus *Helicobacter pylori* in peptic ulcer disease.**

Koch's Criteria	<i>Chlamydia pneumoniae</i> in atherosclerosis	<i>Helicobacter pylori</i> in peptic ulcer disease
Microorganism always present in the diseased tissue	Not always	Not always
Viable microorganism could be cultured from the diseased tissue	Yes (not always)	Yes (not always)
Inoculation of microorganism into susceptible animal would produce disease	Yes	Yes
Microorganism could be detected in the pathological tissue from diseased animal	Yes	Yes

## 1.8. Concluding comments

This chapter reviewed the evidence and established that both acute and chronic infections are risk factors for stroke. Specific examples of acute, local and systemic infections caused by various micro-organisms resulting in stroke were discussed. In keeping with the theories of atherogenesis, the infectious hypothesis of atherosclerosis suggested that chronic infections could play an important role in the pathogenesis of atherosclerosis, and hence, clinical ischaemic event such as a stroke. The roles of chronic infections caused by specific micro-organisms such as *Cytomegalovirus*, *Helicobacter pylori*, dental pathogens and *Chlamydia pneumoniae* in the context of atherosclerosis or stroke were discussed in details. However, the main focus of the discussion was on *C pneumoniae* as it was the most widely studied ‘model’ micro-organism in the context of infectious hypothesis of atherosclerosis. Currently, the concordance of evidence and definitive causal evidence for the involvement of *C pneumoniae* in atherosclerosis are considered lacking. [Ieven and Hoymans, 2005] However, the evidence that infection can be a stimulus for atherogenesis and atherothrombosis continues to mount. [Anderson JL, 2005] The next chapter shall introduce a case-control study on the seroprevalence of *C pneumoniae* in elderly stroke / transient ischaemic attack and control medical patients, from which further research studies submitted for this thesis, were based.

## **Chapter 2**

**An introduction to the ‘*Chlamydia pneumoniae* in elderly patients with stroke’ (C-PEPS) study**

## 2.1. Introduction

As reviewed in chapter 1, the link between *Chlamydia pneumoniae* and atherosclerosis or cerebrovascular disease has been investigated from a number of seroepidemiological, pathological, antibiotic intervention, and carotid doppler imaging studies [Ngeh et al, 2003]. Specifically, the seroepidemiological studies of *C pneumoniae* infection in patients with overt stroke or transient ischaemic attack (TIA) have also been investigated [Wimmer et al, 1996] [Cook et al, 1998] [Fagerberg et al, 1999] [Glader et al, 1999] [Elkind et al, 2000] [Kawashima and Kawada, 2000] [Heuschmann et al, 2001] [Madre et al, 2002] [Tanne et al, 2003] [Bucurescu et al, 2003] [Sirmatel et al, 2003] [Kawamoto et al, 2003] [Anzini et al, 2004] [Johnsen et al, 2005] [Elkind et al, 2006] [Njamnshi et al, 2006] [Piechowski-Jozwiak et al, 2007] [Alamowitch et al, 2008] . The association remains inconclusive with conflicting findings. [Ngeh et al, 2003] [Palm and Grau, 2007]

Most of the studies on the role of *C pneumoniae* in atherosclerotic vascular disease have focussed on younger age groups of patients, and almost entirely excluded the elderly people [Ngeh, 2000]. It is known that most stroke events occur in older people aged 65 to 89 years [Wolf, 1998], and compared with myocardial infarction, stroke patients are at least 10 years older [Warlow, 1998]. The aim of the '*Chlamydia pneumoniae* in elderly patients with stroke' or C-PEPS study was to investigate whether serological markers of *C pneumoniae* infection were associated with acute stroke or TIA in hospital patients aged 65 years or older.

## **2.2. Subjects and Methods**

The C-PEPS study was a case-control study conducted at Whipps Cross University Hospital, a 750-bed acute district general hospital, situated in north-east London, United Kingdom. The period of investigation was from 20 December 1999 to 31 March 2000. [Ngeh et al, 2003]

In this study, stroke was defined clinically as 'a syndrome of rapidly developing clinical signs of focal or global disturbance of cerebral function, with symptoms lasting 24 hours or longer or leading to death, with no apparent cause other than of vascular origin'. [World Health Organisation, 1978] Events lasting less than 24 hours were considered as transient ischaemic attacks. Patients were eligible for inclusion if they were white (to ensure genetic / ethnic homogeneity), aged 65 years or older, and admitted acutely to the hospital under the care of general physicians or geriatricians. [Ngeh et al, 2003]

Exclusion criteria included known immunodeficiency, hypergammaglobulinaemia, connective tissue disease, and other autoimmune disease, as they may interfere with immunoglobulin production and analysis. The exclusion criteria for the controls were history of stroke or TIA, acute or active cardiopulmonary or infective conditions on admission. [Ngeh et al, 2003]

One hundred consecutive subjects aged over 65 years admitted to the Departments of Geriatric and General Medicine, with a primary diagnosis of acute stroke or TIA, were recruited prospectively. Concurrently, 87 control subjects aged over 65 years,

with no past history of stroke or TIA, and admitted with acute non-cardiopulmonary, non-infective disorders to the same medical departments were recruited consecutively. As there was no evidence of active or acute cardiovascular, pulmonary, or infective disease in the controls, there was no reason to suspect that the controls were more predisposed to acquire *C pneumoniae* infection. [Ngeh et al, 2003]

A standardised interview was performed with patients, carers or family doctors in order to obtain a demographic history, and a profile of risk factors for vascular disease such as history of hypertension, smoking habit (current, former: >3 months, never, unknown), diabetes mellitus, hypercholesterolaemia, ischaemic heart disease (IHD), stroke or TIA. Where clinically indicated, computed tomography (CT) brain scan, electrocardiography (ECG), and routine blood tests, were performed under the requests made by clinicians who were not directly involved in the recruitment of patients. [Ngeh et al, 2003]

### **2.3. Serological analysis**

Up to 2 millilitres of each patient's serum obtained on admission was stored at -20°C for subsequent analysis. A commercial kit (SeroCP, Savyon Diagnostics Limited, Israel) was used for the analysis of *C pneumoniae* antibodies. The SeroCP™ is an Enzyme Linked Immunosorbent Assay (ELISA) used for the detection of *C pneumoniae* specific IgG, IgA and IgM antibodies in serum. The antigens used are derived from purified *C pneumoniae* elementary bodies (TW™ 183). Serum samples intended for *C pneumoniae* IgM testing were pre-treated with manufacturer's serum diluent which contains anti-human IgG, to remove Rheumatoid factor and reduce IgG

interference. Sera were analysed independently by two investigators blinded to patients' case or control status. [Ngeh et al, 2003]

## **2.4. Statistical methods**

From two previous case-control studies involving mainly younger patients [Wimmer et al, 1996] [Cook et al, 1998], the odds ratio for *C pneumoniae* infection and cerebrovascular disease was 2 and 4 respectively, and the rate of exposure (seroprevalence) on average was 50% and 30% respectively. A two-sided 5% test ( $Z_{\alpha/2} = 1.96$ ), required to detect a relative risk (or odds ratio as an approximate) of 3 ( $R = 3$ ), with 90% power ( $Z_b = 1.28$ ) was used for sample size calculation. [Woodward, 1992] [Ngeh et al, 2003] Because *C pneumoniae* seroprevalence increases with age [Kanamoto et al, 1991] [Wang et al, 1993] [Paltiel et al, 1995], an exposure rate of 60% ( $p = 0.6$ ) in London's elderly population was assumed, for the purpose of the calculation. [Ngeh et al, 2003] As equal numbers of cases and controls were intended for the sample, case:control ratio of r:1 ( $r = 1$ ) subjects were to be recruited in the study. [Woodward, 1992] [Ngeh et al, 2003] Substituting the above data into a validated statistical formula [Woodward, 1992], a study size of 89 cases and 89 controls was calculated. [Ngeh et al, 2003]

Raw data collected were initially computed using the Microsoft Excel programme. Using the SAS statistical package, a logistic regression model controlling for age, sex, history of hypertension, smoking habit, diabetes mellitus, hypercholesterolaemia, IHD and ischaemic ECG, was constructed to analyse the association of *C pneumoniae* antibody prevalence and stroke or TIA. Odds ratios expressing the associations of

stroke or TIA with *C pneumoniae* IgA, IgG, IgM, and the corresponding 95% confidence intervals (CIs), and P-values, were derived. [Ngeh et al, 2003]

## **2.5. Ethics committee approval**

The study was approved by the Local Research Ethics Committee, Redbridge and Waltham Forest Health Authority, London. Informed consent was obtained from every participant or his / her next of kin. [Ngeh et al, 2003]

## **2.6. Results**

The age and sex distribution were similar in the stroke / TIA and control groups. The median age was 80 years in both groups (range= 65to 98 in cases, 65 to 95 in controls, P= 0.16). Fifty-nine cases (59%) and 60 controls (69%) were females, P= 0.17. [Ngeh et al, 2003]

Among the 100 acute stroke (90) or TIA (10) patients, 64 subjects were admitted with first ever presentation of acute stroke / TIA. Thirty-five subjects had one or more episodes of stroke / TIA in the past. No data for previous stroke / TIA was available for 1 case. None of the subjects was recruited more than once in the study. [Ngeh et al, 2003]

The acute medical conditions of the control patients on admission were grouped as: 1. gastrointestinal (40 subjects), 2. musculoskeletal pain / immobility / falls (20 subjects),



3. haematological (12 subjects), 4. neurological / psychiatric (11 subjects), 5. renal / metabolic disturbances (4 subjects). [Ngeh et al, 2003]

Computed tomography (CT) head scan results were available for 80 of the 100 stroke / TIA patients. Of the 80 stroke / TIA patients who had had CT head scan, 50 were found to have an infarct, 6 had a haemorrhage, 2 a haemorrhagic infarct, 2 a haemorrhage with old infarct, and in 20 no lesion was demonstrated. [Ngeh et al, 2003]

Table 1 shows the distribution of vascular risk factors in stroke / TIA cases and controls. [Ngeh et al, 2003]

**Table 1. Distribution of risk factors in stroke or TIA cases and controls**

<b>Confounder / vascular risk factor</b>		<b>Number (%)</b>	<b>Number (%)</b>	<b>Number (%)</b>
		<b>of cases</b>	<b>of controls</b>	<b>of all subjects</b>
<b>History of hypertension</b>	No	50 (51)	52 (60)	102 (55)
	Yes	49 (49)	35 (40)	84 (45)
	n	99	87	186
<b>Smoking status</b>	Never	37 (38)	29 (33)	66 (36)
	Former	43 (44)	47 (54)	90 (48)
	Current	18 (18)	11 (13)	29 (16)
	n	98	87	185
<b>History of diabetes mellitus</b>	No	91 (91)	77 (89)	168 (90)
	Yes	9 (9)	10 (11)	19 (10)
	n	100	87	187
<b>History of raised cholesterol</b>	No	7 (54)	8 (67)	15 (60)
	Yes	6 (46)	4 (33)	10 (40)
	n	13	12	25
	Missing	87	75	162
<b>History of ischaemic heart disease</b>	No	83 (83)	63 (72)	146 (78)
	Yes	17 (17)	24 (28)	41 (22)
	n	100	87	187
<b>ECG ischaemia</b>	No	42 (44)	38 (54)	80 (48)
	Yes	54 (56)	32 (46)	86 (52)
	n	96	70	166

As shown in Table 2, the seroprevalence of *C pneumoniae* antibodies IgA, IgG, and IgM in the stroke / TIA group was similar to the control group. [Ngeh et al, 2003]

**Table 2. Seroprevalence of *Chlamydia pneumoniae* antibody in cases and controls**

<i>Chlamydia pneumoniae</i> antibody		Number (%) of cases	Number (%) of controls	P-value
<b>Ig A</b>	Negative	33 (33)	29 (33)	P= 1.0
	Borderline	4 (4)	4 (5)	
	Positive	63 (63)	54 (62)	
	Total	100	87	
<b>Ig G</b>	Negative	27 (27)	26 (30)	P= 0.53
	Borderline	2 (2)	4 (5)	
	Positive	71 (71)	57 (65)	
	Total	100	87	
<b>Ig M</b>	Negative	80 (80)	68 (78)	P= 0.78
	Borderline	6 (6)	4 (5)	
	Positive	14 (14)	15 (17)	
	Total	100	87	

The ORs of having a stroke or TIA compared to not having a stroke / TIA in relation to *C pneumoniae* seropositivity were presented in table 3 and were not statistically significant. [Ngeh et al, 2003]

**Table 3. Odds ratios for stroke or TIA in relation to serological markers of *C pneumoniae* infection**

	<b>Odds ratio</b>	<b>95% confidence Interval</b>	<b>P-value from logistic regression</b>
<b>Unadjusted<sup>1</sup></b>			
<b>Ig A</b>	1.04	0.57-1.88	1.00
<b>Ig G</b>	1.29	0.69-2.39	0.53
<b>Ig M</b>	0.78	0.35-1.73	0.78
<b>Adjusted for age and sex</b>			
<b>Ig A</b>	1.02	0.55-1.87	0.96
<b>Ig G</b>	1.25	0.67-2.35	0.48
<b>Ig M</b>	0.79	0.35-1.76	0.56
<b>Adjusted for history of high blood pressure, smoking, diabetes and IHD</b>			
<b>Ig A</b>	0.94	0.51-1.74	0.86
<b>Ig G</b>	1.24	0.65-2.35	0.51
<b>Ig M</b>	0.78	0.33-1.83	0.57

<b>Adjusted for history of high blood pressure, smoking, diabetes, IHD and high cholesterol<sup>2</sup></b>			
<b>Ig A</b>	0.58	0.05-6.35	0.66
<b>Ig G</b>	0.52	0.05-5.18	0.58
<b>Ig M</b>	0.87	0.06-13.25	0.92
<b>Adjusted for history of high blood pressure, smoking, diabetes, IHD, age and sex</b>			
<b>Ig A</b>	0.90	0.47-1.69	0.74
<b>Ig G</b>	1.17	0.61-2.27	0.63
<b>Ig M</b>	0.81	0.34-1.92	0.63
<b>Adjusted for history of high blood pressure, smoking, diabetes, IHD, age, sex and ischaemic ECG</b>			
<b>Ig A</b>	1.04	0.52-2.05	0.92
<b>Ig G</b>	1.24	0.61-2.53	0.55
<b>Ig M</b>	0.79	0.32-1.96	0.60

<sup>1</sup> The P values for this analysis came from a Fisher's exact test.

<sup>2</sup> This analysis was only based on 25 subjects since most subjects had missing data for cholesterol history (see table 1).

The seroprevalence of *C pneumoniae* in the 64 cases who presented with first ever stroke / TIA was 64% for IgA, 73% for IgG, and 17% for IgM. In comparison, the 35 subjects who presented with a recurrent stroke / TIA had a seroprevalence of 60% for *C pneumoniae* IgA, 66% for IgG, and 6% for IgM. The seroprevalence of *C pneumoniae* in the stroke / TIA patients whose CT head scan showed infarction (50 subjects) or no evidence of stroke (20 subjects) was 63% for IgA, 67% for IgG, and 16% for IgM. In the 6 cases whose CT head scan showed only a haemorrhage, the *C pneumoniae* seroprevalence was 83%, 100%, 17% for IgA, IgG, IgM, respectively. [Ngeh et al, 2003]

In order to eliminate any potential influence of IHD on the seroprevalence of *C pneumoniae*, a subgroup of 43 (28 females) acute stroke / TIA patients (median age= 80) and 44 (31 females) control medical patients (median age= 80), who had no known history of IHD or ischaemic ECG or both, were identified in the study retrospectively. Forty-two percent of stroke / TIA patients and 34% of control medical patients had history of hypertension. 36% of stroke / TIA patients and 34% of control medical patients never smoked. 14% of both stroke / TIA patients and control medical patients had diabetes. The seroprevalence of *C pneumoniae* IgA, IgG, and IgM were 67%, 77%, and 19% respectively in stroke / TIA patients, and 61%, 59%, and 14% respectively in control medical patients. The ORs were 1.40 for IgA (95% CI 0.53 to 3.65; P= 0.49), 2.41 for IgG (95% CI 0.90 to 6.46; P= 0.08), 1.55 for IgM (95% CI 0.45 to 5.40; P= 0.49), after adjusting for history of hypertension, smoking, diabetes mellitus, age and sex. [Ngeh et al, 2003]

## 2.7. Discussion

This case-control study conducted in a busy, large district general hospital in London was the first to recruit elderly hospital patients prospectively to examine for the seroprevalence of *C pneumoniae*, and its relationship with acute stroke / TIA. [Ngeh et al, 2003]

In contrast to numerous studies that had reported the seroepidemiology of *C pneumoniae* in the context of ischaemic heart disease [Ngeh et al, 2003], relatively few studies had investigated the association specifically between *C pneumoniae* and overt cerebrovascular disease. Apart from several prospective [Glader, 1999] [Tanne et al, 2003] and case-control [Heuschmann et al, 2001] [Sirmatel et al, 2003] [Alamowitch et al, 2008] studies, most case-control [Wimmer et al, 1996] [Cook et al, 1998] [Elkind et al, 2000] [Kawashima and Kawada, 2000] [Madre et al, 2002] [Bucurescu et al, 2003] [Kawamoto et al, 2003] [Anzini et al, 2004] [Johnsen et al, 2005] [Elkind et al, 2006] [Njamnshi et al, 2006] [Piechowski-Jozwiak et al, 2007] and one prospective [Fagerberg et al, 1999] studies demonstrated a positive association between serological markers of *C pneumoniae* infection and stroke / TIA. The median age of the stroke / TIA and control medical patients in the C-PEPS study was 80 years for both groups, and they were among the oldest age groups ever studied in this clinical context. [Ngeh et al, 2003]

The C-PEPS study utilised an ELISA method for the detection of *C pneumoniae* antibody. Unlike the gold-standard microimmunofluorescence (MIF) assay for *C pneumoniae* serology, the ELISA serological method has only been used in a few

seroepidemiological studies of *C pneumoniae* in the setting of stroke. [Kawashima and Kawada, 2000] [Kawamoto et al, 2003] [Tanne et al, 2003] [Johnsen et al, 2005] [Piechowski-Jozwiak et al, 2007]. The ELISA test is less subjective or operator dependent than the MIF assay [Ngeh and Gupta, 2000]. The overall agreement between MIF and SeroCP tests, according to the manufacturer, was 98% for IgA, 95% for IgG, and 89% for IgM. The results of ELISA were reported as positive, borderline or negative on the basis of a single dilution of a serum sample, and not as an endpoint titre. Nevertheless, like the MIF assay, its reproducibility, sensitivity, and specificity would need further clarification. [Ngeh et al, 2003]

The presence of *C pneumoniae* IgA, IgG, or IgM in the serum generally reflects recurrent / persistent, chronic / past, or acute infection, respectively [Ngeh and Gupta, 2001]. However, *C pneumoniae* IgA may not be a reliable marker, and increased level should not be taken as evidence for ongoing chronic infection [Tompkins et al, 2000]. Serological markers of chronic *C pneumoniae* infection in the C-PEPS study were found to be much more prevalent than in the other UK study [Cook et al, 1998], and reached a seroprevalence of 71% of cases and 65% of controls for IgG antibody. This is in keeping with reports from other countries that *C pneumoniae* seropositivity increases with age [Kanamoto et al, 1991] [Wang et al, 1993] [Paltiel et al, 1995], with a 50 to 70% prevalence of seropositivity in middle-aged adults [Leinonen, 1993]. In contrast, a few studies report declining *C pneumoniae* IgG titres with age. [Einarsson et al, 1994] [Coles et al, 1999] Most adults are infected two to three times during their lifetime [Leinonen, 1993], and increasing seropositivity with age is suggestive of chronic infection or reinfection throughout life. [Ngeh et al, 2003]



The seroprevalence of *C pneumoniae* in stroke / TIA patients did not differ significantly from controls (Table 2). Among the stroke / TIA patients, there was no significant difference when comparing the seroprevalence of *C pneumoniae* between patients presenting with first ever and recurrent stroke / TIA. The inclusion of the very few haemorrhagic stroke patients in the study might weaken any association between *C pneumoniae* infection and stroke, since atherosclerosis could be an important common link. However, as previously reported [Cook et al, 1998], the seroprevalence of *C pneumoniae* in patients suffering from ischaemic and haemorrhagic strokes were similar. [Ngeh et al, 2003]

The unadjusted ORs for having an acute stroke / TIA, and IgA, IgG and IgM seropositivity were 1.04, 1.29 and 0.78, respectively (Table 3). Using a logistic regression statistical model, the ORs were adjusted for age and sex, and essentially remained unchanged. After adjustment for history of hypertension, smoking, diabetes, IHD, age and sex, the ORs for having an acute stroke or TIA were 0.90, 1.17 and 0.81 for IgA, IgG and IgM seropositivity, respectively, and the corresponding P-values were not significant. The ORs remained 1.04, 1.24, 0.79 (P= NS), essentially unchanged even after adjusting for the presence of ischaemic ECG. These results suggested that serological markers of *C pneumoniae* infection were not significantly associated with acute stroke / TIA. The ORs after adjustment for hypercholesterolaemia revealed an inverse relationship between *C pneumoniae* infection and stroke or TIA, which was unexpected. This could be an artefact due to the analysis which was based on only 25 subjects, since data for cholesterol history was not available in most subjects (see Table 1). [Ngeh et al, 2003]

Ischaemic heart disease has been associated with *C pneumoniae* in seroepidemiological studies [Ngeh and Gupta, 2001]. To exclude any possible influence of IHD on the seroprevalence of *C pneumoniae*, 43 acute stroke / TIA cases (median age= 80) and 44 controls (median age= 80) without any history of IHD or ischaemic ECG were identified. There was a trend towards increased ORs for acute stroke or TIA, especially for IgG (OR= 2.41, CI 0.90-6.46, P= 0.08), even after statistical adjustment. This suggested that *C pneumoniae* specific IgG may be weakly associated with acute cerebrovascular disease in a subgroup of elderly patients without any known history of IHD / ischaemic ECG. [Ngeh et al, 2003] This implied that chronic or previous *C pneumoniae* infection may serve as a link between inflammation and atherogenesis or atherothrombosis in cerebrovascular disease [Ngeh et al, 2003]. However, a firm conclusion was limited by the reduced sample size.

The C-PEPS study was conducted during the winter months of December to March, which were usually associated with a high incidence of respiratory tract infection in the elderly population [Woodhouse et al, 1994]. Subclinical infections or even recent epidemics might temporarily induce a high antibody response in the control subjects, and thereby obscure any genuine serological association between *C pneumoniae* infection and stroke / TIA [Ngeh, 2000].

To minimise background differences in cases and controls, suitable hospital elderly patients (often with a number of comorbidities and cardiovascular risk factors) were recruited as controls. Although the controls gave no clinical history of stroke or TIA, silent cerebrovascular disease in this group could not be excluded as atherosclerosis is commonly associated with ageing [Stout, 1998]. Therefore a link between subclinical

atherosclerosis and *C pneumoniae* infection as reflected by its high seroprevalence in the elderly population is a possibility [Ngeh, 2000]. However, this could also explain the negative results of this study. [Ngeh et al, 2003] CT brain scan would be useful to exclude asymptomatic stroke in the elderly control subjects in future studies. Moreover, high *C pneumoniae* seroprevalence could simply reflect life-long recurrent or chronic infection- an infective burden without any aetiological link with atherosclerosis [Ngeh, 2000].

The C-PEPS study confirmed a high seroprevalence of *C pneumoniae* antibody levels in older patients. [Ngeh et al, 2003] A significant association between serological markers of *C pneumoniae* infection and acute cerebrovascular events in an elderly hospital population was not found. [Ngeh et al, 2003] However, there was a weak trend towards increased ORs for acute stroke / TIA in a subgroup of elderly patients without any history of IHD or ischaemic ECG. [Ngeh et al, 2003]

## **2.8. Aims of further published works and thesis**

The patients recruited for the C-PEPS study have allowed further original research opportunities and resulted in published works submitted for this thesis. The aims of the published works submitted for this thesis are summarised as follows:

1. To investigate the reproducibility of SeroCP, an enzyme-linked immunosorbent assay (ELISA) commercial kit used to detect *Chlamydia pneumoniae*-specific antibodies in the C-PEPS study

2. To establish the seroprevalences of other atypical respiratory pathogens such as *Mycoplasma pneumoniae*, *Legionella pneumophila*, *Coxiella burnetii* in elderly stroke / TIA and medical patients
3. To conduct a case-control study on the relationship between seropositivity of individual atypical respiratory pathogen and elderly stroke / TIA versus medical patients
4. To conduct a case-control study on the relationship between an aggregate number of chronic infectious burden of atypical respiratory pathogens and elderly stroke / TIA versus medical patients
5. To conduct a case-control study on the prevalence of electrocardiographic rhythms and ischaemic changes in elderly stroke / TIA and medical patients
6. To conduct a case-control study on the relationship between socioeconomic deprivation, atypical respiratory infections and survival outcome in elderly stroke / TIA and medical patients

The next chapter will report a study on the reproducibility of SeroCP enzyme-linked immunosorbent assay (ELISA) used in the detection of *C. pneumoniae*-specific antibodies in the C-PEPS study.

## **Chapter 3**

**The reproducibility of SeroCP enzyme-linked immunosorbent assay (ELISA) for detection of *Chlamydia pneumoniae*-specific antibodies**

### 3.1. Introduction

As discussed in previous chapters, the association between *Chlamydia pneumoniae* and atherosclerotic vascular diseases has been investigated extensively. These investigations include sero-epidemiological observations, pathological specimen examinations, animal models, immunological / molecular studies, and antibiotic intervention studies [Ngeh et al, 2002]. Among these diversified areas of research, sero-epidemiology was the first and most commonly used method in this context. [Ngeh et al, 2004(a)]

Most of the *C pneumoniae* sero-epidemiological studies have used the technique of microimmunofluorescence (MIF). A few have used enzyme-linked immunosorbent assay (ELISA) tests to investigate the relationship between *C pneumoniae* seropositivity and stroke. [Kawashima and Kawada, 2000] [Tanne et al, 2003] [Johnsen et al, 2005] [Piechowski-Jozwiak et al, 2007] [Ngeh et al, 2004(a)] However, data on the reproducibility of ELISA used in *C pneumoniae* serology is limited. This study aimed to investigate the reproducibility of SeroCP commercial ELISA kits used in the detection of *C pneumoniae*-specific antibodies. [Ngeh et al, 2004(a)]

### 3.2. Patients and Methods

The sera used in this study were obtained from a consecutive series of 187 elderly acute stroke / TIA patients and control medical patients (median age= 80 years) recruited prospectively in the C-PEPS study as described in previous chapter. [Ngeh et al, 2003] Patient's sera were stored at -20 °C before analysis.

SeroCP commercial ELISA kits, manufactured by Israeli company Savyon Diagnostics Limited, in accordance with GMP standards and ISO-9002 certification for the production and sale of diagnostics, were used for serological analysis. The antigens in these ELISA kits originated from purified *C pneumoniae* elementary bodies (TW 183), and enabled specific and sensitive detection of *C pneumoniae* immunoglobulin (Ig) A, IgG, and IgM. [Ngeh et al, 2004(a)]

Sera for IgM testing were pre-treated with the manufacturer's serum diluent which contained anti-IgG, to remove rheumatoid factor and reduce IgG interference. Each serum was diluted at a 1:105 dilution with the supplied serum diluent before analysis, as recommended by the manufacturer. The case or control status of the sera was blinded during analyses. These were performed by two experienced investigators following the manufacturer's instructions. Due to a limited availability of the SeroCP ELISA kits, only the first 122 consecutive patients' sera were examined for *C pneumoniae* IgA and IgG, and 138 for IgM. The serological tests were then repeated, and the results analysed to establish the reproducibility of these SeroCP ELISA kits. [Ngeh et al, 2004(a)]

The determination and validation of the test results were in accordance with the manufacturer's instructions. The absorbance or optical density (OD), proportional to the amount of specific antibodies bound to the coated antigens in each plate, was read at a wave-length of 450nm by a photometric machine (Organon Teknika Reader 530, Version 1.20). For a valid test, two criteria must be met: (1) the OD<sub>450</sub> of the positive

control at 450nm should be  $\geq 0.8$ ; and (2) the mean OD<sub>450</sub> of the negative control (NC) at 450nm should be  $> 0.1$  but  $\leq 0.4$ . [Ngeh et al, 2004(a)]

In order to normalise the results from different tests, a cut-off index (COI) was calculated according to the formula:  $\text{COI} = \text{serum sample OD}_{450} \div \text{cut-off value (COV)}$ ; and where the COV was calculated using the formula:  $\text{COV} = \text{twice the mean of two negative control OD}_{450} \text{ readings}$ . As defined by the manufacturer, a COI of  $< 1.0$  means negative (n) result (i.e. no detectable antibodies), a COI of 1 to 1.1 means borderline (b) result (i.e. low level of antibodies), and a COI of  $> 1.1$  means positive (p) result (i.e. relevant level of antibodies). [Ngeh et al, 2004(a)]

The manufacturer suggested that if borderline results were obtained from two specimens taken from the same patient 2 to 4 weeks apart, the specimens should be considered negative. However, in this study, second serum samples were not collected from patients giving borderline results. Therefore, a single borderline result was defined as negative, as the low level of antibodies detected was not sufficient to be considered positive. [Ngeh et al, 2004(a)]

### **3.3. Results**

The COI of positive samples ranged from 1.15 to 6.16 for *C pneumoniae* IgA, from 1.12 to 4.91 for IgG, and from 1.12 to 5 for IgM. In the repeat tests or second run, the COI of positive samples ranged from 1.14 to 5.23 for IgA, 1.101 to 3.45 for IgG, and from 1.20 to 3.49 for IgM. [Ngeh et al, 2004(a)]



Table 1 on the following page shows the COI values of samples with borderline results in one run and their corresponding COI in another run. [Ngeh et al, 2004(a)]

The mean Cut-Off Index of all borderline samples in the first and second runs versus their mean COI in the corresponding second and first runs were similar for IgA and IgM: 1.06 versus 1.01 for IgA (9 samples in each run), 1.04 versus 0.89 for IgM (12 samples). They were 1.08 versus 1.89 for IgG, but the comparison was made on only 3 samples that gave borderline COI.

**Table 1a. Cut-off index (COI) of samples with borderline results in the first run and their corresponding COI in the second run**

Sample	IgA 1 <sup>st</sup> run	2 <sup>nd</sup> run	Sample	IgG 1 <sup>st</sup> run	2 <sup>nd</sup> run	Sample	IgM 1 <sup>st</sup> run	2 <sup>nd</sup> run
37	1.05(b)	0.46(n)	107	1.08(b)	2.12(p)	17	1.00(b)	0.99(n)
39	1.09(b)	0.83(n)	115	1.08(b)	1.70(p)	25	1.10(b)	0.45(n)
56	1.04(b)	0.69(n)				30	1.08(b)	0.53(n)
74	1.09(b)	1.17(p)				45	1.09(b)	0.63(n)
99	1.10(b)	0.50(n)				54	1.04(b)	0.92(n)
104	1.05(b)	1.17(p)				60	1.04(b)	0.97(n)
						83	1.06(b)	1.04(b)
						114	1.03(b)	1.23(p)
						119	1.00(b)	1.57(p)
<b>Mean</b>	<b>1.07</b>	<b>0.80</b>		<b>1.08</b>	<b>1.91</b>		<b>1.05</b>	<b>0.93</b>

**Table 1b. Cut-Off Index (COI) of samples with borderline results in the second run and their corresponding COI in the first run**

Sample	IgA 2 <sup>nd</sup> run	1 <sup>st</sup> run	Sample	IgG 2 <sup>nd</sup> run	1 <sup>st</sup> run	Sample	IgM 2 <sup>nd</sup> run	1 <sup>st</sup> run
10	1.02(b)	1.85(p)	80	1.07(b)	1.85(p)	83	1.04(b)	1.06(b)
78	1.08(b)	1.27(p)				136	1.02(b)	0.71(n)
110	1.03(b)	1.17(p)				138	1.01(b)	0.54(n)
<b>Mean</b>	<b>1.04</b>	<b>1.43</b>					<b>1.02</b>	<b>0.77</b>

**Table 1c. Mean Cut-Off Index (COI) of samples with borderline results in either the first or second runs and mean of their corresponding COI**

	Mean of all borderline COI	Mean of all corresponding COI
<b>IgA</b>	<b>1.06</b>	<b>1.01</b>
<b>IgG</b>	<b>1.08</b>	<b>1.89</b>
<b>IgM</b>	<b>1.04</b>	<b>0.89</b>

The COI of those samples with discrepant results are given in Table 2. [Ngeh et al, 2004(a)] The mean COI of the discrepant samples were 1.39 versus 0.97 for IgA (15 samples in each run), 1.43 versus 1.21 for IgG (20 samples), 1.32 versus 0.80 for IgM (14 samples). The differences in the mean COI of these discrepant samples were small: 0.42 for IgA, 0.22 for IgG, 0.52 for IgM. [Ngeh et al, 2004(a)]

**Table 2. Cut-Off Index of samples giving discrepant results in the first and second runs**

<b>Sample</b>	<b>IgA</b>		<b>Sample</b>	<b>IgG</b>		<b>Sample</b>	<b>IgM</b>	
	<b>1st run</b>	<b>2<sup>nd</sup> run</b>		<b>1<sup>st</sup> run</b>	<b>2<sup>nd</sup> run</b>		<b>1<sup>st</sup> run</b>	<b>2<sup>nd</sup> run</b>
10	1.85(p)	1.02(b)	5	0.93(n)	1.29(p)	2	1.22(p)	0.46(n)
17	1.37(p)	0.71(n)	17	1.47(p)	0.79(n)	3	1.21(p)	0.55(n)
28	1.51(p)	0.75(n)	21	1.44(p)	0.90(n)	6	1.58(p)	0.60(n)
44	1.15(p)	0.40(n)	47	2.04(p)	0.68(n)	8	1.23(p)	0.64(n)
74	1.09(b)	1.17(p)	48	0.83(n)	1.28(p)	24	1.12(p)	0.78(n)
78	1.27(p)	1.08(b)	50	3.56(p)	0.80(n)	27	1.29(p)	0.61(n)
80	1.67(p)	0.98(n)	56	0.80(n)	1.96(p)	29	2.52(p)	0.91(n)
91	1.15(p)	0.66(n)	65	1.13(p)	0.59(n)	31	1.93(p)	0.70(n)
95	1.40(p)	0.89(n)	66	4.13(p)	0.39(n)	37	1.25(p)	0.65(n)
96	2.64(p)	0.95(n)	80	1.85(p)	1.07(b)	41	1.19(p)	0.66(n)
98	1.63(p)	0.71(n)	90	2.71(p)	0.78(n)	42	1.25(p)	0.66(n)
104	1.05(b)	1.17(p)	95	0.71(n)	1.43(p)	98	0.64(n)	1.20(p)
110	1.17(p)	1.03(b)	97	0.60(n)	1.44(p)	114	1.03(b)	1.23(p)
117	0.96(n)	1.16(p)	102	0.80(n)	1.17(p)	119	1.00(b)	1.57(p)
122	0.89(n)	1.81(p)	106	0.76(n)	1.25(p)			
			107	1.08(b)	2.12(p)			
			113	0.96(n)	1.101(p)			
			115	1.08(b)	1.70(p)			
			117	0.92(n)	1.38(p)			
			122	0.89(n)	2.12(p)			
<b>Mean</b>	<b>1.39</b>	<b>0.97</b>		<b>1.43</b>	<b>1.21</b>		<b>1.32</b>	<b>0.80</b>

The results of SeroCP ELISA reproducibility are summarised in Table 3. [Ngeh et al, 2004(a)] For the detection of *C pneumoniae* IgA, IgG, and IgM, the first and repeated runs of ELISA tests gave different results i.e. one negative / borderline, one positive, or vice versa, in 15/122, 20/122 and 14/138 samples, respectively. The percentage (%) sample disagreement and 95% confidence interval (CI) were 12% (CI= 6 to 18%), 16% (CI= 9 to 23%), and 10% (CI= 5 to 15%) for *C pneumoniae* IgA, IgG and IgM, respectively. The reproducibility of ELISA used for the detection of *C pneumoniae* IgA, IgG, and IgM, expressed as Kappa values, were 0.73, 0.60, and 0.53, respectively (P < 0.001). [Ngeh et al, 2004(a)]

**Table 3. SeroCP ELISA Reproducibility**

<i>Chlamydia pneumoniae</i> Antibody	The first and second runs of ELISA			% sample disagreement (95% CI)	Kappa value (P value)
	Both tests negative	Both tests positive	One test negative or borderline, one positive		
Ig A (n= 122)	36	71	15	12% (15/122) (6-18%)	0.73 (p < 0.001)
Ig G (n= 122)	25	77	20	16% (20/122) (9-23%)	0.60 (p < 0.001)
Ig M (n= 138)	114	10	14	10% (14/138) (5-15%)	0.53 (p < 0.001)

### 3.4. Discussion and conclusions

The results in Table 3 show a good sample agreement of > 80%, which is similar to that reported for MIF tests [Peeling et al, 2000]. The Kappa value quantifies the agreement between the first and repeated ELISA tests, after allowing for random variation and the number of observations used [Siegel and Castellan, 1988]. A Kappa value of 0 indicates no agreement, whereas a value of 1.0 indicates perfect agreement. If  $0.61 < \text{Kappa} < 0.80$ , good concordance is indicated, while  $0.41 < \text{kappa} < 0.60$  indicates a moderate concordance. The kappa values for SeroCP ELISA's reproducibility were 0.73 for *C pneumoniae* IgA, 0.60 for IgG, and 0.53 for IgM, and these were statistically significant with  $P < 0.001$ . [Ngeh et al, 2004(a)]

Serological testing is considered the most useful means of determining the prevalence of *C pneumoniae* infection in epidemiological studies [Dowell et al, 2001]. The MIF is currently the reference standard in *C pneumoniae* serology, but it is subjective and requires an expert microscopist to interpret the results [Dowell et al, 2001] [Ossewaarde et al, 2000]. The interlaboratory variation or reproducibility of MIF tests among 14 research laboratories has been assessed by testing 22 identical sera, and the overall percentage agreement with the reference laboratory is around 60% to 80%. [Peeling et al, 2000] [Ngeh et al, 2004(a)] [Ieven and Hoymans, 2005] In addition to the subjective issue, other factors such as the type, purity, and concentration of the antigen used and the assay procedure might contribute to the disagreement between the MIF tests. [Ieven and Hoymans, 2005]

The antigens in the SeroCP ELISA kits originated from purified *C pneumoniae* elementary bodies (TW 183), and enabled specific and sensitive detection of *C pneumoniae* immunoglobulin (Ig) A, IgG, and IgM. [Ngeh et al, 2004(a)] In contrast, earlier ELISA that used *Chlamydia* genus-specific lipopolysaccharides (LPS) as the antigen can cross-react non-specifically with other *Chlamydia* species. [Ossewaarde et al, 2000] Indeed, cross-reactivity between *C pneumoniae* and other *Chlamydia* species has been demonstrated with the MIF test. [Ieven and Hoymans, 2005] [Apfalter, 2006]

In comparison, ELISA is a more objective test than MIF [Dowell et al, 2001] [Ieven and Hoymans, 2005]. The SeroCP ELISA result is qualitative, and is reported on the basis of a single 1:105 dilution of a serum sample, and not as an endpoint titre. It can be automated by a photometric machine, with the advantages of high throughput and electronic records. ELISA is therefore easier to standardise and is the preferred diagnostic method in routine laboratories [Ossewaarde et al, 2000]. Because ELISAs can afford a relatively high throughput and objectivity, they are increasingly used in seroepidemiological studies. [Ngeh and Gupta, 2004] [Ngeh et al, 2004(a)]

In the clinical setting, a single ELISA test result should not be used for a final diagnosis. Serum samples obtained early during primary infection may not contain detectable antibodies. If *C pneumoniae* infection is suspected, a second sample should be obtained 2 to 4 weeks later and tested in parallel with the original sample. However, all clinical and laboratory data should be considered when making a diagnosis. [Ngeh et al, 2004(a)] Traditionally, the interpretation of serological results should be based on a combination of *C pneumoniae* IgA, IgG and IgM profiles. [Ngeh et al, 2004(a)] A combination of positive IgM, negative or positive IgG, and negative or positive IgA

results indicate current *C pneumoniae* infection. A positive IgG and a combination of negative IgM and IgA results indicate past or current infection. A positive IgA, with positive or negative IgG and negative IgM would refer to current or chronic infection. Although unvalidated, others have commented that IgA antibodies, which are produced for only 3 to 5 days after exposure, are a marker of persistent, chronic infection; whereas IgG antibodies, which remain elevated for several years after infection, are a marker of remote, completed infection. [Elkind et al, 2006] [Dowell et al, 2001] As the seroprevalences of *C pneumoniae* in the general population are high, whether seropositivity for *C pneumoniae* results either from a chronic, active infection or from a past infection, remains an unresolved issue. [Ieven and Hoymans, 2005]

According to the SeroCP ELISA kit manufacturer, when sera obtained from 55 suspected patients attending a respiratory disease clinic and 33 healthy individuals were tested using in-house MIF and SeroCP ELISA, the overall agreement between the two tests were 98% for IgA, 95% for IgG, 89% for IgM. Others have also reported a moderately good correlation between MIF results and ELISA (LabSystems, Helsinki, Finland) tests ( $r= 0.8$ ,  $P= 0.001$ ) [Ossewaarde et al, 2000] [Messmer et al, 2001]. Similarly, as reported in other studies [Persson and Boman, 2000] [Numazaki et al, 1996], the sensitivity and specificity for ELISA when compared to MIF were reported as around 90% by the manufacturer. Subsequent study using ELISA test (Viracell, Spain) to detect *C pneumoniae* IgG has demonstrated 100% sensitivity and 85% specificity, and good concordance with the MIF test. [Gutierrez et al, 2002]

A previous study has reported good reproducibility for LabSystems ELISA kit for the detection of *C pneumoniae* IgG. The mean and median coefficients of variation were

reported as 10.2% and 8.6% respectively [Messmer et al, 2001]. As recommended, the accuracy of new diagnostic kit such as SeroCP ELISA's reproducibility needs proper evaluation, and this was the focus of the current study. [Ngeh et al, 2004(a)] The present study was the first to evaluate and report the reproducibility of SeroCP ELISA kits used to detect *C pneumoniae* specific IgA, IgG and IgM antibodies simultaneously, in the C-PEPS study that involved over a hundred elderly acute stroke and general medical patients. [Ngeh et al, 2004(a)] Subsequent study confirmed a good agreement between SeroCP ELISA and Labsystems MIF tests for the detection of *C pneumoniae* IgG ( $r= 0.93$ ;  $P= 0.0008$ ) and IgA ( $r= 0.72$ ;  $P= 0.00072$ ), in patients with coronary heart disease. [Ciervo et al, 2004]

Several studies have demonstrated that the link between *C pneumoniae* and coronary artery disease depends on the serologic method chosen to measure the *C pneumoniae* antibodies. [Apfalter, 2006] [Schumacher A et al, 2001] [Hoymans et al, 2003] [Maraha et al, 2004] Because *C pneumoniae* is an intracellular pathogen, there is a poor correlation between direct detection e.g. by culture / polymerase chain reaction, and serology. For example, in two multicentre pneumonia treatment studies, it was shown that only 1% to 3% of the culture positive patients met the serologic criteria and around 70% with positive cultures for *C pneumoniae* were seronegative. [Hammerschlag 2000] [Apfalter, 2006] Whether the results obtained by SeroCP ELISA might correlate with endovascular chronic infection remains controversial. [Ngeh et al, 2004(a)]

Nevertheless, preliminary data have suggested that ELISA has a good sensitivity, specificity, and reproducibility when compared to MIF. [Ngeh and Gupta, 2004]



[Ngeh et al, 2004(a)] ELISA may become a preferred, objective test used in the sero-epidemiological study of *C pneumoniae* infection and its link with atherosclerotic vascular disease. However, further efforts to standardise new commercially available ELISA kits against MIF tests, and to express their results in international units will be required [Dowell et al, 2001] [Tuuminen et al, 2000(a)].

This study concluded that SeroCP ELISA had a good reproducibility for IgA, and a moderately good reproducibility for IgG and IgM. Although SeroCP ELISA was comparable to MIF, its level of reproducibility was not perfect and this fact should be considered when using such a kit for further epidemiological or diagnostic study. [Ngeh et al, 2004(a)] Indeed, the use of ELISA in seroepidemiological studies of atypical respiratory or other pathogens in the setting of stroke / TIA should be considered in similar context relating to the various methodological issues concerning *C pneumoniae* serology and atherosclerosis.

The next chapter will present the ‘*Mycoplasma pneumoniae* in elderly patients with stroke’ or M-PEPS study which was based on the same cohort of patients as in the C-PEPS study.

## Chapter 4

***Mycoplasma pneumoniae* in elderly patients with stroke: a case-control study on the seroprevalence of *Mycoplasma pneumoniae* in elderly patients with acute stroke / transient ischaemic attack. The M-PEPS study**

#### 4.1. Introduction

As discussed in previous chapters, infections are recognised as risk factors for stroke [Elkind and Cole, 2006] [Palm and grau, 2007]. Specifically, *Chlamydia pneumoniae* is an atypical respiratory pathogen that has been linked to atherosclerotic vascular diseases [Kalayoglu et al, 2002] [Ngeh et al, 2002]. *Mycoplasma pneumoniae*, another atypical respiratory micro-organism, has similar epidemiological behaviour and ability to cause extra-pulmonary manifestations and chronic sequelae following respiratory tract infection [Taylor-Robinson, 1996] [Taylor-Robinson and Thomas, 1998]. In fact, a link between *M pneumoniae* and ischaemic heart disease (IHD) has been investigated in several seroepidemiological [Pönkä et al, 1981] [Gurfinkel et al, 1997] [Horne et al, 2000] [Reunanen et al, 2002] [Momiyama et al, 2004] [Goyal et al, 2007] and pathological [Maraha et al, 2000] [Higuchi et al, 2000] [Maraha et al, 2001] [Higuchi and Ramires, 2002] [Higuchi et al, 2003] [Gois et al, 2006] studies. The results of these investigations were largely supportive of a relationship between *M pneumoniae* infection and IHD or atherosclerosis.

Specifically, a positive association between *M pneumoniae* infection and cerebrovascular disease has also been reported [Ode and Gronberg, 1976] [Arthur and Margolis et al, 1977] [Parker et al, 1981] [Nakahata et al, 1983] [Mulder et al, 1987] [Dowd et al, 1987] [Mulder and Spierings et al, 1987] [Visudhiphan et al, 1992] [Fu et al, 1998] [Benito-Leon et al, 1998] [Ganesan et al, 1999] [Padovan et al, 2001] [Ovetchkine et al, 2002] [Antachopoulos et al, 2002] [Leonardi et al, 2005] [Greco et al, 2006] [Tanir et al, 2006]. [Sotgiu et al, 2003] [Tsiodras et al, 2005] These reports are limited to a number of clinical case studies involving mainly paediatric and

younger patients. As age is one of the most important risk factors for cerebrovascular disease [Wolf, 1998], the aim of the present study was to establish for the first time whether serological markers of *M pneumoniae* infection were associated with acute stroke / transient ischaemic attack (TIA) versus control medical patients aged 65 years or older. [Ngeh et al, 2004(b)]

## **4.2. Subjects and Methods**

The findings of the C-PEPS (*Chlamydia pneumoniae* in elderly patients with stroke) case-control study were presented in Chapter 2 [Ngeh et al, 2003]. The present '*Mycoplasma pneumoniae* in elderly patients with stroke' or M-PEPS study was a case-control study nested within the C-PEPS study. The M-PEPS study had 10 fewer patients than the C-PEPS. [Ngeh et al, 2004(b)]

The investigation took place at Whipps Cross University Hospital in north-east London, during the period 20 December 1999 to 31 March 2000. The inclusion and exclusion criteria of the patients in the M-PEPS study were similar to that in the C-PEPS study as described previously. [Ngeh et al, 2003] [Ngeh et al, 2004(b)]

Ninety-five consecutive patients admitted with a primary diagnosis of acute stroke / TIA and 82 control patients admitted with acute non-cardiopulmonary, non-infective medical conditions were included in the present study. Patients' demographic history and profile of vascular risk factors such as history of hypertension, smoking, diabetes mellitus, hypercholesterolaemia, IHD, stroke or TIA were obtained. Investigations such as computed tomography (CT) brain scan, electrocardiography (ECG), and

routine blood tests, were performed under the requests made by clinicians not directly involved in the recruitment of patients. [Ngeh et al, 2004(b)]

#### **4.2.1. Serological Analysis**

Up to 2 ml of each patient's serum obtained on admission was stored at -20<sup>0</sup>C for subsequent analysis at the end of the study period. Three separate commercial enzyme-linked immunosorbent assay (ELISA) kits: SeroMP-IgA, SeroMP-IgG, SeroMP-IgM, manufactured by Savyon Diagnostics Limited, Israel, were used for serological analysis. [Ngeh et al, 2004(b)] The microtitre plates of the SeroMP kits were coated with a purified fraction of *M pneumoniae* P1-enriched membrane antigens to ensure specific detection of *M pneumoniae* specific IgA, IgG and IgM antibodies. Serum samples intended for *M pneumoniae* IgM testing were pre-treated to remove rheumatoid factor and reduce IgG interference. Two experienced investigators blinded to patients' case or control status were involved in serological analysis in accordance to standard manufacturer's instruction. [Ngeh et al, 2004(b)]

The SeroMP ELISA result was determined on the basis of a single 1:105 dilution of a serum sample and absorbance value i.e. optical density (OD) measured by an ELISA reader (Organon Teknika Reader 530, Version 1.21) at a wavelength of 450/620 nm. The absorbance value (OD) was proportional to the level of specific *M pneumoniae* antibody tested. Using a graph paper, a linear curve was plotted with 3 validated OD values (generated from 3 calibrators) on the Y-axis against their corresponding concentrations of 10, 50, 100 BU/ml (binding units per ml of serum) on the X-axis. By using this standard curve, the level of specific antibody expressed as BU/ml in the

tested sample was interpolated from each absorbance or OD measured. According to the manufacturer's instruction, values of  $< 10$  BU/ml were considered negative or no detectable antibody,  $\geq 10$  BU/ml were considered positive or relevant level of antibody, and  $\geq 50$  BU/ml were considered high positive or high level of antibody. [Ngeh et al, 2004(b)]

#### **4.2.2. Statistical methods**

The sample size calculation generating a size of 89 cases and 89 controls in the original C-PEPS study has been described in chapter 2. [Ngeh et al, 2003]. Using the SAS statistical package, a logistic regression model controlling for history of hypertension, smoking, diabetes mellitus, age and sex, history of IHD and ischaemic ECG was constructed to determine the association of *M pneumoniae* antibody prevalence and stroke / TIA. The odds ratios (ORs) expressing the associations of stroke or TIA with *M pneumoniae* IgA, IgG and IgM, and the corresponding 95% confidence intervals (CIs), and P values, were derived. [Ngeh et al, 2004(b)]

#### **4.2.3. Ethics committee approval**

This study was approved by the Local Research Ethics Committee, Redbridge and Waltham Forest Health Authority, London. Informed consent was obtained from every patient or his / her next of kin.

### 4.3. Results

The median age was 80 years in both groups (range= 65 to 98 in cases, 66 to 95 in controls). Fifty-six stroke / TIA patients (59%) and 57 control medical patients (70%) were females. Among the cases (85 stroke and 10 TIA cases), 62 subjects were admitted with their first ever acute stroke or TIA. Thirty-two subjects had history of stroke or TIA in the past. In 1 case, past history of stroke or TIA was not available. [Ngeh et al, 2004(b)]

The acute medical conditions of the control patients on admission were grouped as: (1) gastrointestinal (38 subjects), (2) musculoskeletal pain / immobility / falls (20 subjects), (3) haematological (12 subjects), (4) neurological / psychiatric (8 subjects), (5) renal / metabolic disturbances (4 subjects). [Ngeh et al, 2004(b)]

CT head scan results were available for 76 of the 95 cases. Of the 76 stroke / TIA patients who had had CT head scan, 48 were found to have an infarct, 6 had a haemorrhage, 2 a haemorrhagic infarct, 1 a haemorrhage with old infarct, and in 19 no lesion was demonstrated. These data were not entirely new as they have been published [Ngeh et al, 2003] and discussed in chapter 2 of this thesis. [Ngeh et al, 2004(b)]

Table 1 shows the distribution of vascular risk factors in 95 stroke / TIA patients and 82 control medical patients. This was similar to that in C-PEPS study as the sample sizes in both studies were almost identical.

**Table 1. Distribution of risk factors in 95 stroke / TIA cases and 82 controls**

<b>Confounder / vascular risk factor</b>		<b>Cases (%)</b>	<b>Controls (%)</b>
<b>History of hypertension</b>	No	47 (50)	50 (61)
	Yes	47 (50)	32 (39)
	n	94	82
<b>Smoking habits</b>	Never	35 (38)	26 (32)
	Former	40 (43)	45 (55)
	Current	18 (19)	11 (13)
	n	93	82
<b>History of diabetes mellitus</b>	No	86 (91)	73 (89)
	Yes	9 (9)	9 (11)
	n	95	82
<b>History of raised cholesterol</b>	No	6 (50)	8 (67)
	Yes	6 (50)	4 (33)
	n	12	12
	Missing	83	70
<b>History of ischaemic heart disease</b>	No	79 (83)	60 (73)
	Yes	16 (17)	22 (27)
	n	95	82
<b>ECG ischaemia</b>	No	41 (45)	38 (58)
	Yes	50 (55)	28 (42)
	n	91	66



As shown in table 2, the seroprevalence of *M pneumoniae* antibodies IgA, IgG and IgM in the stroke / TIA group was similar to the control group.

**Table 2. Seroprevalence of *M pneumoniae* antibody in cases and controls**

<i>M pneumoniae</i> antibody		Cases (%)	Controls (%)	P
<b>Ig A</b>	Negative	20 (21)	13 (16)	0.44
	Positive	75 (79)	69 (84)	
	Total	95	82	
<b>Ig G</b>	Negative	37 (39)	41 (50)	0.17
	Positive	58 (61)	41 (50)	
	Total	95	82	
<b>Ig M</b>	Negative	89 (94)	73 (89)	0.29
	Positive	6 (6)	9 (11)	
	Total	95	82	

The ORs of having a stroke / TIA compared to not having a stroke / TIA in relation to *M pneumoniae* seropositivity are presented in table 3 and are not statistically significant.

**Table 3. Odds ratios for stroke / TIA in relation to serological markers of *M pneumoniae* infection**

<i>M pneumoniae</i> antibody	Odds ratio	95% confidence interval	P
<b>Unadjusted for confounders</b>			
IgA	0.71	0.30-1.63	0.44
IgG	1.57	0.83-2.98	0.17
IgM	0.55	0.15-1.82	0.29
<b>Adjusted for history of high blood pressure, smoking, diabetes, age, sex, history of IHD and ischaemic ECG</b>			
IgA	0.63	0.26-1.52	0.31
IgG	1.32	0.66-2.64	0.43
IgM	0.52	0.14-1.92	0.32

In order to examine for any potential influence of IHD on the seroprevalence of *M pneumoniae*, a subgroup of 42 (27 females) acute stroke / TIA patients (median age= 80) and 43 (30 females) control medical patients (median age= 80), who had no known history of IHD or ischaemic ECG (defined as the presence of abnormal Q-wave patterns, ST segment depression or elevation, T-wave inversion or flattening, left bundle branch block) or both, were identified in the study retrospectively. The seroprevalence of *M pneumoniae* IgA, IgG and IgM were 83%, 52% and 2% respectively in cases, and 84%, 47% and 12% respectively in controls. The ORs were 0.82 for IgA (95% CI 0.24 to 2.78; P= 0.75), 1.16 for IgG (95% CI 0.47 to 2.85; P= 0.75), 0.18 for IgM (95% CI 0.02 to 1.73; P= 0.14), after adjusting for history of hypertension, smoking, diabetes mellitus, age and sex.

#### **4.4. Discussion**

In the context of ischaemic heart disease (IHD), *M pneumoniae* antibodies have been found more frequently in cases of unstable angina than in controls [Gurfinkel et al, 1997]. *M pneumoniae* antibodies IgA [Horne et al, 2000] and IgG [Reunanen et al, 2002] were shown to predict the diagnosis of IHD and incidence of coronary events respectively. A Japanese study also reported that *M pneumoniae* seropositivity was associated with coronary artery disease (CAD), but only in patients with *C pneumoniae* seropositivity (odds ratio= 5.1; 95% CI= 1.8 to 14.9) [Momiya et al, 2004] Recently, a study has found that combined seropositivity to *C pneumoniae* and *M pneumoniae* was significantly higher ( $P < 0.05$ ) in CAD patients with myocardial infarction (MI) than in those without MI. [Goyal et al, 2007] Indeed, *M pneumoniae* has also been detected in atherosclerotic specimens, along with *C pneumoniae*.

[Maraha et al, 2000] [Higuchi et al, 2000] [Maraha et al, 2001] [Higuchi and Ramires, 2002] [Higuchi et al, 2003] [Gois et al, 2006] [Higuchi et al, 2006] The *Mycoplasmas* were initially found mainly in the lipid core of the ruptured thrombosed plaque, since vulnerable atheromas were rich in cholesterol which the micro-organisms depended for growth and survival. [Higuchi et al, 2000]

It has been suggested that co-infection by *M pneumoniae* and *C pneumoniae* may represent an important co-factor for plaque instability and rupture, leading to coronary plaque thrombosis and acute myocardial infarction; since larger amount of these bacteria was strongly correlated with histological signs of more plaque vulnerability, thrombosis, rupture, and inflammation. [Higuchi et al, 2000] [Higuchi and Ramires, 2002] [Higuchi et al, 2003] [Gois et al, 2006] However, *M pneumoniae* and *C pneumoniae* antigens were also found to be present in early atheromas, and a higher *M pneumoniae* to *C pneumoniae* ratio was correlated with increased growth factors, lower inflammation and plaque stability. [Higuchi et al, 2006] In contrast, a predominance of *C pneumoniae* in relation to *M pneumoniae* was found to favour progression of the plaque, which was associated with increased B-cell proliferation. [Gois et al, 2006]

Although *M pneumoniae* was known to cause central nervous system diseases [Taylor-Robinson et al, 1996] [Sotgiu et al, 2003] [Greenlee et al, 2000] [Tsiodras et al, 2005], specific association between *M pneumoniae* and acute cerebrovascular disease was limited to case reports involving mainly younger patients [Arthur and Margolis et al, 1977] [Ode and Gronberg, 1976] [Parker et al, 1981] [Nakahata et al, 1983] [Mulder et al, 1987] [Dowd et al, 1987] [Mulder and Spierings et al, 1987]

[Visudhiphan et al, 1992] [Fu et al, 1998] [Benito-Leon et al, 1998] [Padovan et al, 2001] [Ovetchkine et al, 2002] [Antachopoulos et al, 2002] [Leonardi et al, 2005] [Greco et al, 2006] [Tanir et al, 2006]. [Sotgiu et al, 2003] [Tsiodras et al, 2005]

This M-PEPS case-control study was the first to recruit elderly hospital patients prospectively to examine for the seroprevalence of *M pneumoniae*, and its relationship with acute stroke or TIA. [Ngeh et al, 2004(b)] The study utilised SeroMP commercial ELISA kits. According to the manufacturer, these kits had been validated and used in the serodiagnosis of *M pneumoniae* infection, and were found to have good sensitivity (92% for IgA, 89% for IgG, 80% for IgM) and specificity (74% for IgA, 90% for IgG, 82% for IgM); as reported in the literature. [Watkins-Riedel et al, 2001] [Lieberman et al, 2002] [Beersma et al, 2005]. The SeroMP ELISA results were semi-quantitative and not based on specific cut-off antibody titres. [Ngeh et al, 2004(b)]

The interpretation of serological results should be based on a combination of IgA, IgG and IgM antibodies' profile. The presence of *M pneumoniae* IgA, IgG or IgM in the serum generally reflects current / recurrent, past / current or current infection, respectively. [Ngeh et al, 2004(b)] The serological markers of *M pneumoniae* infection in the M-PEPS study were found to be much more prevalent than in other studies [Rastawicki et al, 1998] [Hauksdottir et al, 1998] [Tuuminen et al, 2000(b)] [Daxboeck et al, 2002], and reached a seroprevalence of 79% of cases and 84% of controls for IgA, 61% of cases and 50% of controls for IgG, and 6% of cases and 11% of controls for IgM antibodies. [Ngeh et al, 2004(b)]

Previous studies in the UK have reported that during the outbreaks of *M pneumoniae* infection, infection confirmed by laboratory tests was least common in adults over 65 years of age. [Rastawicki et al, 1998] [Noah, 1976] [Noah and Urquhart, 1980] [Ghosh and Clements, 1992] Other studies have reported that older patients (over 50 years of age) have significantly lower *M pneumoniae* seropositivity rate or antibody titres than younger patients. [Hauksdottir et al, 1998] [Daxboeck et al, 2002] However, in a healthy Finnish population study using quantitative enzyme immunoassays, the seroprevalence of *M pneumoniae* was found to level off at about 40% to 50% in adulthood, and the prevalence did not exceed 60% for IgG or 35% for IgA in subjects aged over 65 years. [Tuuminen et al, 2000(b)]

Despite a high *M pneumoniae* seroprevalence in this M-PEPS study, it is interesting to note that there was no clinical evidence of acute or serious *M pneumoniae* respiratory tract infection especially in the control patients recruited in this study. This may be explained by subclinical or asymptomatic infections in up to 15% of cases [Daxboeck et al, 2002]; or related to a problem with test specificity. [Ngeh et al, 2004(b)]

The seroprevalence of *M pneumoniae* in stroke / TIA patients did not differ significantly from controls (Table 2). The unadjusted ORs for having an acute stroke / TIA in relation to IgA, IgG and IgM seropositivity were 0.71, 1.57 and 0.55, respectively (Table 3). Using a logistic regression statistical model, the ORs were adjusted for history of hypertension, smoking, diabetes, age and sex, history of IHD and ischaemic ECG. The adjusted ORs for having an acute stroke or TIA were 0.63, 1.32 and 0.52 for IgA, IgG, IgM seropositivity, respectively, and the corresponding P values were not significant. These results suggested that serological markers of *M*

*pneumoniae* infection were not significantly associated with acute stroke / TIA. The ORs were not adjusted for history of hypercholesterolaemia as the data for cholesterol history were not available in most patients (Table 1). [Ngeh et al, 2004(b)]

The inclusion of haemorrhagic stroke or thromboembolic stroke arising from valvular heart disease in the study might weaken any association between *M pneumoniae* infection and ischaemic stroke, since atherosclerosis could be an important aetiological link. [Ngeh et al, 2004(b)] However, only 6 out of the 95 cases had a radiological confirmation of a haemorrhagic stroke without any new or old infarct. For these 6 cases, the *M pneumoniae* seropositivity was 83.3% (positive in 5/6 cases), 66.7% (4/6 cases) and 16.7% (1/6 cases) for IgA, IgG and IgM, respectively, and these seroprevalences were in fact higher than those in ischaemic stroke cases. Hence, the inclusion of these 6 cases would not have lessened the ability of the study to detect any specific association between *M pneumoniae* seropositivity and atherothrombotic stroke. Interestingly, seropositivity of *C pneumoniae* infection had been reported to associate with haemorrhagic stroke [Cook et al, 1998]. Data on the prevalence of valvular heart disease in the cases and controls were not available. However, using echocardiography to exclude specific thromboembolic stroke arising from valvular heart disease in the cases (i.e. non-atherosclerotic stroke) may be helpful in future studies. [Ngeh et al, 2004(b)]

As discussed, IHD has been associated with *M pneumoniae* infection. To examine for any possible influence of IHD on the seroprevalence of *M pneumoniae*, 42 acute stroke / TIA patients (median age= 80) and 43 control medical patients (median age= 80) without any history of IHD or ischaemic ECG were identified in the M-PEPS

study retrospectively. After adjustment for history of hypertension, smoking, diabetes, age and sex, the ORs did not suggest any association between *M pneumoniae* serological markers and acute stroke / TIA. This is in contrast to the C-PEPS study whereby a trend towards increased ORs for acute stroke or TIA was observed, which suggested that *C pneumoniae* serological markers may be weakly associated with acute cerebrovascular disease in this subgroup of patients without any known history of IHD or ischaemic ECG. [Ngeh et al, 2003] [Ngeh et al, 2004(b)]

This M-PEPS study had several limitations. Only the first 177 of 187 consecutive series of cases and controls recruited concurrently in the C-PEPS study were included in this M-PEPS study, due to limited availability of ELISA kits. Like the C-PEPS study, this M-PEPS study was powered to detect an OR of  $\geq 3.0$  [Ngeh et al, 2003]. Therefore, while this study might rule out *M pneumoniae* seropositivity as a major risk factor for stroke in this elderly population, a smaller effect could not be excluded or detected due to the small sample size. A much larger study would be required to effectively rule out *Mycoplasma* infection as a risk factor for stroke. Silent or subclinical cerebrovascular disease in the control patients could not be excluded, although CT brain scan would have been helpful to detect asymptomatic stroke in this group. Subclinical *M pneumoniae* infection or even recent winter epidemic during the study period (December 1999 to March 2000) might temporarily induce a high antibody response in the control subjects, thereby obscuring any genuine serological association between *M pneumoniae* infection and stroke or TIA. [Ngeh et al, 2004(b)]

However, it is likely that a high *M pneumoniae* seroprevalence in the elderly population simply reflect life-long recurrent or chronic infection without any



significant relationship with atherosclerosis. [Ngeh et al, 2004(b)] It is interesting to note that most published cases [Tsiodras et al, 2005] reporting strokes associated with acute *M pneumoniae* infection occurred mainly in paediatric or younger age groups of patients- a population with much less atherosclerotic burden. Indeed, the mechanisms postulated to link *M pneumoniae* infection and stroke in these younger patients included focal vasculitis, direct invasion of vessel wall, post-infectious autoimmune process (molecular mimicry), and intravascular hypercoagulable state, leading to cerebrovascular thromboembolic phenomena. [Ngeh et al, 2004(b)] [Greenlee and Rose, 2000] [Sotgiu et al, 2003] [Tsiodras et al, 2005] In fatal cases, cerebral pathology frequently combines capillary thromboses and microangiopathy, perivenular lymphocytic infiltrations and demyelination in perivascular location [Sotgiu et al, 2003]. [Ngeh et al, 2004(b)]

#### **4.5. Conclusions**

The M-PEPS study revealed a high seroprevalence of *M pneumoniae* in older stroke / TIA and medical patients in hospital using ELISA. Although the study ruled out *M pneumoniae* seropositivity as a major risk factor for stroke in this elderly population, a smaller effect could not be excluded due to the small sample size. Future adequately powered studies may be required to clarify and define the precise role of *M pneumoniae* infection as a potential factor in the pathogenesis of different subtypes of ischaemic stroke, in all age groups, and in different ethnic populations. [Ngeh et al, 2004(b)]

In the next chapter, results of the '*Legionella pneumophila* in elderly patients with stroke' or L-PEPS study will be presented. The L-PEPS study was also based on the same cohort of patients as in the C-PEPS study. A case-control analysis on the aggregate number of chronic infectious burden of atypical respiratory pathogens in elderly patients will also be presented and discussed.

## **Chapter 5**

***Chlamydia pneumoniae*, *Mycoplasma pneumoniae* and *Legionella pneumophila* in elderly patients with stroke (C-PEPS, M-PEPS, L-PEPS): a case-control study on the infectious burden of atypical respiratory pathogens in elderly patients with acute stroke / transient ischaemic attack**

## 5.1. Introduction

Multiple studies have suggested acute and chronic respiratory infections as potential risk factors for stroke. [Lavalley et al, 2002] [Paganini-Hill et al, 2003] [Linsberg et al, 2003] As discussed in previous chapters, the link between atypical respiratory pathogens such as *Chlamydia pneumoniae* and *Mycoplasma pneumoniae* and cerebrovascular disease has been investigated in seroepidemiological, pathological and clinical case studies. [Ngeh et al, 2003] [Ngeh et al, 2004(b)] Recent studies have reported an association between exposure to a number of infectious pathogens, i.e. infectious burden, and the occurrences and prognoses of atherosclerotic vascular diseases. [Zhu et al, 2000] [Rupprecht et al, 2001] [Zhu et al, 2001] [Auer et al, 2002] [Bloemenkamp et al, 2002] [Espinola-Klein et al, 2002(a)] [Espinola-Klein et al, 2002(b)] [Horne et al, 2002] [Prasad et al, 2002] [Pugh et al, 2002] [Basinkevich et al, 2003] [Cotter et al, 2003] [Smieja et al, 2003] [Lobzin et al, 2005] [Corrado et al, 2006] These studies mainly focussed on ischaemic heart disease (IHD) and were performed mainly in younger patients. [Ngeh and Goodbourn, 2005(a)] However, negative results have also emerged. [Ridker et al, 1999] [Choussat et al, 2000] [Schiele et al, 2001] [Haider et al, 2002] [Khairy et al, 2003] [Rothenbacher et al, 2003] [Niu et al, 2005] [Hagiwara et al, 2007]

*Legionella pneumophila*, like *C pneumoniae* and *M pneumoniae*, is an atypical respiratory pathogen. [Poshni and Millian, 1985] [File et al, 1998] The aim of the present study was to investigate whether serological markers or immunoglobulins (Igs) of *L pneumophila* infection and the aggregate number (infectious burden) of *C pneumoniae*, *M pneumoniae* and *L pneumophila* seropositivity were associated with

acute stroke / transient ischaemic attack (TIA) in subjects aged 65 years or older.  
[Ngeh and Goodbourn, 2005(a)]

## **5.2. Subjects and Methods**

The C-PEPS (*Chlamydia pneumoniae* in elderly patients with stroke) and M-PEPS (*Mycoplasma pneumoniae* in elderly patients with stroke) case-control studies were reported in previous chapters. [Ngeh et al, 2003] [Ngeh et al, 2004(b)] The C-PEPS had 100 cases and 87 controls, whereas the M-PEPS had 95 cases and 82 controls. The subjects of the current '*Legionella pneumophila* in elderly patients with stroke' or L-PEPS case-control study originated from the C-PEPS study. The L-PEPS study had excluded 7 cases of haemorrhagic stroke without acute infarction as detected on computed tomography (CT) head scan. Additionally, the L-PEPS study had excluded 2 cases and 1 control because of a shortage of enzyme-linked immunosorbent assay (ELISA) kits. Thus, the L-PEPS study consisted of the remaining 91 cases and 86 controls. The seroprevalence of *L pneumophila* IgG was measured for all of them, but that of *L pneumophila* IgM was measured for only 81 cases and 72 controls because of a shortage of IgM ELISA kits. Of the 91 cases and 86 controls of the L-PEPS study, the seroprevalences of *C pneumoniae*, *M pneumoniae*, and *L pneumophila* were measured for only 88 cases and 82 controls for IgG and for 81 cases and 72 controls for IgM because of a limited supply of ELISA kits. All studies were approved by the hospital's local research ethics and research and development committees. [Ngeh and Goodbourn, 2005(a)]

The study population was recruited at a 750-bed acute district general hospital in north-east London from 20 December 1999 to 31 March 2000. [Ngeh et al, 2003] All patients' demographics and history of vascular risk factors such as hypertension, smoking, diabetes mellitus, hypercholesterolaemia, IHD, stroke / TIA were obtained. Investigations such as CT brain scan, electrocardiography (ECG), and routine blood tests were ordered / performed by clinicians not directly involved in the recruitment of patients. The inclusion and exclusion criteria of the patients were similar to that in the C-PEPS study. [Ngeh et al, 2003] [Ngeh and Goodbourn, 2005(a)]

### **5.2.1. Serological analysis**

Up to 2 millilitres of each patient's serum obtained on admission was stored at -20°C for subsequent analysis at the end of the study period. A commercial ELISA kit (Vircell SL, Granada, Spain) was used for the analysis of *L pneumophila* serogroup 1-specific IgG and IgM antibodies in serum. The antigens used are derived from inactivated LPS antigens of *L pneumophila* serogroup 1. Sera intended for *L pneumophila* IgM testing were pre-treated with manufacturer's anti-IgG sorbent to remove rheumatoid factor and reduce IgG interference. The results of the ELISA were interpreted as negative, equivocal, or positive on the basis of a single dilution of a serum sample and optical density measured by a spectrophotometer at a wavelength of 450/620 nm. Sera were analysed independently by two investigators blinded to the case or control status of the patients, in accordance with the manufacturer's protocol. [Ngeh and Goodbourn, 2005(a)]

### 5.2.2. Statistical methods

The sample size calculation was based on the C-PEPS study, which had 90% power to detect an odds ratio (OR) of  $\geq 3.0$ . [Ngeh et al, 2003] Raw data were initially computed with the use of the Microsoft Excel programme. With the use of the SAS statistical package, a logistic regression model adjusting for confounders such as age, sex, hypertension, smoking, diabetes mellitus, hypercholesterolaemia, IHD and ischaemic ECG was constructed to analyse the association of *L pneumophila* serogroup 1 antibody and stroke / TIA. Cases and controls that were tested IgG or IgM seropositive for either 0, 1, 2, or all 3 pathogens were identified. Fisher's exact tests were used for crude analysis, and logistic regression was used to adjust for confounders. The ORs for stroke / TIA in relation to infectious burden were determined. Specific combinations of *C pneumoniae*-*M pneumoniae*, *M pneumoniae*-*L pneumophila*, or *L pneumophila*-*C pneumoniae* IgG seropositivity and the risk of stroke / TIA were also investigated. [Ngeh and Goodbourn, 2005(a)]

### 5.3. Results

In this L-PEPS study, the median age was 80 years (range= 65 to 98 years) in the 91 cases and 82 years (range= 65 to 95 years) in the 86 controls. Fifty-three cases (58%) and 59 controls (69%) were females. CT head scan results were available for 71 of the 91 cases: 49 had an infarct, 2 had a haemorrhagic infarct, and in 20 no lesion was demonstrated. [Ngeh and Goodbourn, 2005(a)]

Table 1 shows the distribution of vascular risk factors in 91 stroke / TIA cases and 86 controls. [Ngeh and Goodbourn, 2005(a)]

**Table 1. Distribution of risk factors in 91 stroke / TIA cases and 86 controls\***

<b>Vascular risk factor</b>	<b>Cases (%)</b>	<b>Controls (%)</b>
<b>Hypertension</b>		
No	47 (52)	51 (59)
Yes	43 (48)	35 (41)
N	90	86
<b>Smoking</b>		
Never	34 (38)	28 (32)
Former	41 (45)	47 (55)
Current	15 (17)	11 (13)
N	90	86
<b>Diabetes mellitus</b>		
No	83 (91)	76 (88)
Yes	8 (9)	10 (12)
N	91	86
<b>Hypercholesterolaemia</b>		
No	5 (50)	8 (67)
Yes	5 (50)	4 (33)
N	10	12
Missing	81	74
<b>Ischaemic heart disease</b>		
No	74 (81)	62 (72)
Yes	17 (19)	24 (28)
N	91	86
<b>ECG ischaemia</b>		
No	38 (44)	38 (54)
Yes	49 (56)	32 (46)
N	87	70

\*Because of missing values, the numbers of cases and controls do not always add to 91 and 86, respectively



The seroprevalences of *L pneumophila* IgG and IgM in the stroke / TIA group were 29% and 12% respectively, and were similar to those in the control group (22% and 10%) (Table 2). [Ngeh and Goodbourn, 2005(a)]

**Table 2. Seroprevalence of *L pneumophila* antibody in cases and controls**

<i>L pneumophila</i>	Cases	Controls	P*
Antibody	n (%)	n (%)	
<b>Ig G</b>			
Negative	52 (57)	57 (66)	0.50
Equivocal	13 (14)	10 (12)	
Positive	26 (29)	19 (22)	
Total	91	86	
<b>Ig M</b>			
Negative	67 (83)	62 (86)	0.84
Equivocal	4 (5)	3 (4)	
Positive	10 (12)	7 (10)	
Total	81	72	

\* From Fisher's exact test

The ORs for stroke / TIA in relation to *L pneumophila* seropositivity were 1.52 (95% CI, 0.70 to 3.28; P= 0.29) for IgG and 1.49 (95% CI, 0.45 to 4.90; P= 0.51) for IgM, after statistical adjustment for potential confounders (Table 3). [Ngeh and Goodbourn, 2005(a)]

**Table 3. Odds ratios for stroke / TIA in relation to serological markers of *L pneumophila* infection**

<i>L pneumophila</i> antibody	Odds ratio <sup>*</sup>	95% confidence interval	P
<b>Unadjusted for confounders</b>			
<b>Ig G</b>	1.41	0.71-2.79	0.32
<b>Ig M</b>	1.31	0.47-3.64	0.61
<b>Adjusted for hypertension, smoking, diabetes, ischaemic heart disease, age, sex, and ischaemic ECG</b>			
<b>Ig G</b>	1.52	0.70-3.28	0.29
<b>Ig M</b>	1.49	0.45-4.90	0.51

\*Odds ratio of being antibody positive compared with antibody negative / equivocal by logistic regression analysis

The ORs for stroke / TIA in relation to the number of pathogens tested seropositive for IgG or IgM are presented in Table 4. The ORs in relation to IgG seropositivity for 1, 2, or 3 atypical respiratory pathogens after adjustment were 3.89 (95% CI, 1.13 to 13.33), 2.00 (95% CI, 0.64 to 6.21), and 6.67 (95% CI, 1.22 to 37.04), respectively (P= 0.06). [Ngeh and Goodbourn, 2005(a)]

**Table 4. Odds ratios for stroke / TIA in relation to IgG or IgM seropositivity for 0, 1, 2 or 3 of *C pneumoniae*, *M pneumoniae*, and *L pneumophila***

Number of pathogens	Number of cases	Number of controls	Odds ratio (crude)	Odds ratio (adjusted)*
<b>IgG</b>				
0	8	16	1	1
1	30	21	2.86 (1.03-7.89) <sup>†</sup>	3.89 (1.13-13.33) <sup>†</sup>
2	39	42	1.86 (0.72-4.82) <sup>†</sup>	2.00 (0.64-6.21) <sup>†</sup>
3	11	3	7.33 (1.58-33.97) <sup>†</sup>	6.67 (1.22-37.04) <sup>†</sup>
All	88	82	P= 0.04 <sup>‡</sup>	P= 0.06 <sup>‡</sup>
The P-value for a test of trend across the odds ratios was 0.10 (crude) and 0.23 (adjusted)				
<b>IgM</b>				
0	60	55	1	1
1	17	10	1.56 (0.66-3.69) <sup>†</sup>	1.44 (0.53-3.91) <sup>†</sup>
2	4	6	0.61 (0.16-2.28) <sup>†</sup>	0.77 (0.17-3.50) <sup>†</sup>
3	0	1	Not estimable	Not estimable
All	81	72	P= 0.87 <sup>‡</sup>	P= 0.91 <sup>‡</sup>
Number of pathogens	Number of first-ever cases	Number of controls	Odds ratio (crude)	Odds ratio (adjusted)*
<b>IgG</b>				
0	5	16	1	1
1	19	21	2.90 (0.89-9.43) <sup>†</sup>	3.80 (0.89-16.39) <sup>†</sup>
2	26	42	1.98 (0.65-6.05) <sup>†</sup>	2.25 (0.59-8.55) <sup>†</sup>
3	7	3	7.47 (1.39-40.25) <sup>†</sup>	7.09 (1.05-47.62) <sup>†</sup>
All	57	82	P= 0.09 <sup>‡</sup>	P= 0.15 <sup>‡</sup>

The P-value for a test of trend across the odds ratios was 0.12 (crude) and 0.20 (adjusted)

\* Adjusted for age, sex, hypertension, diabetes mellitus, smoking, ischaemic heart disease and ischaemic ECG

<sup>†</sup> Numbers in parentheses are 95% confidence intervals

<sup>‡</sup> From logistic regression analysis (test of association between number of pathogens and risk of stroke / TIA)

Table 5 shows the ORs for stroke / TIA in relation to specific combinations of *C pneumoniae*-*M pneumoniae*, *M pneumoniae*-*L pneumophila*, and *L pneumophila*-*C pneumoniae* IgG seropositivity. [Ngeh and Goodbourn, 2005(a)]

**Table 5. Odds ratios for stroke / TIA in relation to specific combinations of *C pneumoniae*-*M pneumoniae*, *M pneumoniae*-*L pneumophila*, and *L pneumophila*-*C pneumoniae* IgG seropositivity**

Combination of pathogens (IgG)	Seropositivity	Odds ratio*	P†
<b>Cp-Mp</b>	No pathogens‡	1.00	0.20
	Either Cp or Mp	2.84 (0.90-8.93)	
	Both Cp and Mp	2.50 (0.79-7.87)	
<b>Mp-Lp</b>	No pathogens‡	1.00	0.47
	Either Mp or Lp	1.29 (0.56-2.95)	
	Both Mp and Lp	2.09 (0.65-6.71)	
<b>Lp-Cp</b>	No pathogens‡	1.00	0.18
	Either Lp or Cp	2.38 (0.80-7.09)	
	Both Lp and Cp	3.22 (0.91-11.36)	

Cp indicates *C pneumoniae*; Mp, *M pneumoniae*; and Lp, *L pneumophila*

Numbers in parentheses are 95% confidence intervals

\* Adjusted for age, sex, hypertension, diabetes mellitus, smoking, ischaemic heart disease and ischaemic ECG

† From logistic regression analysis

‡ Not positive for any of Cp, Mp, or Lp

As described previously, the acute medical conditions of the 87 controls in the C-PEPS study were grouped as follows: (1) gastrointestinal (40 subjects); (2) musculoskeletal pain / immobility / falls (20 subjects); (3) haematological (12 subjects); (4) neurological / psychiatric (11 subjects); and (5) renal / metabolic disturbances (4 subjects). [Ngeh et al, 2003]

The seroprevalences of *C pneumoniae*, *M pneumoniae*, and *L pneumophila* were similar among the medical condition groups of the controls (except *M pneumoniae* and *L pneumophila* IgM; see Table 6). [Ngeh and Goodbourn, 2005(a)]

**Table 6. Seroprevalences of *C pneumoniae*, *M pneumoniae*, and *L pneumophila* in the control medical condition groups**

	Number of Controls	P, Fisher's exact test
	Positive / Total (%)	
<b><i>C pneumoniae</i> IgG</b>		
GI	27 / 40 (68)	0.86
MIF	12 / 20 (60)	
H	9 / 12 (75)	
NP	7 / 11 (64)	
RM	2 / 4 (50)	
All	57 / 87 (66)	
<b><i>M pneumoniae</i> IgG</b>		
GI	19 / 38 (50)	0.39
MIF	7 / 20 (35)	
H	7 / 12 (58)	
NP	6 / 8 (75)	
RM	2 / 4 (50)	
All	41 / 82 (50)	
<b><i>L pneumophila</i> IgG</b>		
GI	8 / 40 (20)	0.97
MIF	5 / 20 (25)	
H	3 / 12 (25)	
NP	2 / 10 (20)	
RM	1 / 4 (25)	
All	19 / 86 (22)	
<b><i>C pneumoniae</i> IgM</b>		
GI	7 / 40 (18)	0.99
MIF	4 / 20 (20)	
H	2 / 12 (17)	
NP	2 / 11 (18)	
RM	0 / 4 (0)	
All	15 / 87 (17)	
<b><i>M pneumoniae</i> IgM</b>		
GI	1 / 38 (3)	0.02
MIF	2 / 20 (10)	
H	4 / 12 (33)	
NP	2 / 8 (25)	
RM	0 / 4 (0)	
All	9 / 82 (11)	
<b><i>L pneumophila</i> IgM</b>		
GI	1 / 32 (3)	0.06
MIF	1 / 17 (6)	
H	3 / 11 (27)	
NP	2 / 8 (25)	
RM	0 / 4 (0)	
All	7 / 72 (10)	

GI indicates gastrointestinal; MIF, musculoskeletal pain / immobility / falls; H, haematological; NP, neurological / psychiatric; and RM, renal / metabolic disturbances

Among the 81 stroke and 10 TIA cases in the L-PEPS study, 58 subjects were admitted with first-ever stroke / TIA. Thirty-two subjects had one or more episodes of stroke / TIA in the past. Table 7 compared the IgG and IgM seroprevalences of *C pneumoniae*, *M pneumoniae*, and *L pneumophila*, as well as their aggregate number in first-ever (including 1 case without data for previous stroke / TIA) and recurrent stroke / TIA cases, showing no significant differences in IgG and IgM status between the first-ever and recurrent cases. [Ngeh and Goodbourn, 2005(a)]



**Table 7. Seroprevalences of *C pneumoniae*, *M pneumoniae*, and *L pneumophila* IgG and IgM and aggregate number of pathogens in first-ever and recurrent stroke / TIA cases**

Pathogens	Seropositivity*	Number of stroke / TIA cases (%)		P <sup>†</sup>
		First ever	Recurrent	
<b>Cp IgG</b>	Negative or equivocal	17 (28)	12 (36)	0.49
	Positive	43 (72)	21 (64)	
	All	60	33	
<b>Mp IgG</b>	Negative or equivocal	23 (40)	11 (35)	0.82
	Positive	34 (60)	20 (65)	
	All	57	31	
<b>Lp IgG</b>	Negative or equivocal	42 (71)	23 (72)	1.00
	Positive	17 (29)	9 (28)	
	All	59	32	
<b>Aggregate of IgG</b>	<b>No. of pathogens</b>			0.98
	0	5 (9)	3 (10)	
	1	19 (33)	11 (35)	
	2	26 (46)	13 (42)	
	3	7 (12)	4 (13)	
	All	57	31	
<b>Cp IgM</b>	Negative or equivocal	49 (82)	31 (94)	0.13
	Positive	11 (18)	2 (6)	
	All	60	33	
<b>Mp IgM</b>	Negative or equivocal	54 (95)	29 (94)	1.00
	Positive	3 (5)	2 (6)	
	All	57	31	
<b>Lp IgM</b>	Negative or equivocal	46 (85)	25 (93)	0.48
	Positive	8 (15)	2 (7)	
	All	54	27	
<b>Aggregate of IgM</b>	<b>No. of pathogens</b>			0.62
	0	38 (70)	22 (81)	
	1	13 (24)	4 (15)	
	2	3 (6)	1 (4)	
	All	54	27	

Cp indicates *C pneumoniae*; Mp, *M pneumoniae*; and Lp, *L pneumophila*

<sup>†</sup> From Fisher's exact test

## 5.4. Discussion

In the L-PEPS study, the seroprevalences of *L pneumophila* serogroup 1 were 12% to 29% for the elderly stroke / TIA patients and 10 to 22% for the elderly control patients, both within the range of 0.4% to 32% reported worldwide. [Poshni and Millian, 1985] Two Singaporean studies have reported *L pneumophila* seroprevalence of between 10% - 22% in the healthy population or blood bank donors, using indirect immunofluorescence test. [Nadarajah et al, 1987] [Heng et al, 1997] Using indirect immunofluorescence technique, the seroprevalence of *L pneumophila* infections (serogroups 1 to 6) among 562 healthy people in Naples, Italy was 23%. [Romano et al, 1989] In this group, there were no significant differences associated with sex and age, even though an apparent increase with age was observed. [Romano et al, 1989] In Chile, a study using similar method among 100 healthy blood donors reported that the seroprevalence of *L pneumophila* (serogroups 1 to 6) was 5%. [Lobos et al, 1993] Using indirect immunofluorescence assay and microagglutination test, another Italian study reported that the prevalence of *L pneumophila* serogroup 1 antibodies in 777 blood donors was only 0.3%. [Franzin and Scramuzza, 1995] Most worldwide seroepidemiological studies showed no age or sex related *L pneumophila* seroprevalence differences, although others reported both a decreasing prevalence in the over 50 years age group in both sexes [Yonke et al, 1982] and a 2.7 times higher prevalence rate in men over 40 years of age compared to women of comparable ages [Helms et al, 1980]. [Poshni and Millian, 1985]

In the L-PEPS study, the seroprevalence of *L pneumophila* in stroke / TIA patients did not differ significantly from that of controls (Table 2). With the use of logistic

regression to adjust for hypertension, smoking, diabetes, IHD, age, sex and ischaemic ECG, the ORs for stroke / TIA in relation to IgG and IgM seropositivity were 1.52 (P= 0.29) and 1.49 (P= 0.51) respectively (Table 3). These results suggested that serological markers of *L pneumophila* infection were not significantly associated with acute stroke / TIA. [Ngeh and Goodbourn, 2005]

Recent studies have suggested that an increasing number of micro-organisms, i.e. infectious burden, as evidenced by serological markers of chronic infection such as IgG, was associated with an incremental risk of cardiovascular and cerebrovascular diseases. [Zhu et al, 2000] [Kiechl et al, 2001] [Rupprecht et al, 2001] [Zhu et al, 2001] [Auer et al, 2002] [Espinola-Klein et al, 2002(a)] [Espinola-Klein et al, 2002(b)] [Horne et al, 2002] [Prasad et al, 2002] [Pugh et al, 2002] [Basinkevich et al, 2003] [Cotter et al, 2003] [Smieja et al, 2003] [Lobzin et al, 2005] [Corrado et al, 2006] A prospective study has reported that chronic bacterial infections e.g. respiratory, urinary tract, dental and others may amplify the risk of atherosclerosis development in the carotid arteries. [Kiechl et al, 2001] The age and sex adjusted odds ratio for any chronic infection versus none was 4.08 (95% CI= 2.42 to 6.85; P< 0.0001) [Kiechl et al, 2001] Further studies have concluded that the number of infectious pathogens (pathogens' IgG or IgA antibodies) or infectious burden e.g. *herpes simplex virus* 1 and 2, *cytomegalovirus*, *Epstein-Barr virus*, *Hemophilus influenzae*, *Chlamydia pneumoniae*, *Mycoplasma pneumoniae*, *Helicobacter pylori* and *Hepatitis A virus*, to which an individual has been exposed was not only an independent factor involved in the development and progression of carotid atherosclerosis [Espinola-Klein et al, 2002(a)] [Espinola-Klein et al, 2002(b)] but also a predictor of an increased hazard of stroke, myocardial infarction or cardiovascular death [Smieja et al, 2003]. Indeed, a

recent prospective follow-up study found that the total burden of infections or IgG seropositivity for *H pylori*, *Cytomegalovirus* and *C pneumoniae* was highly predictive of cerebrovascular or cardiovascular events ( $P < 0.0001$ ). [Corrado et al, 2006]

However, negative reports have emerged. Other studies have reported that IgG and IgA seropositivity or previous infection to more than one micro-organisms such as *C pneumoniae*, *H pylori*, *herpes simplex virus* and *cytomegalovirus* was not associated with an increased risk for stroke, myocardial infarction or cardiovascular death. [Ridker et al, 1999] [Haider et al, 2002] A recent Japanese study reported a low detection rate for *C pneumoniae*, *cytomegalovirus*, *herpes simplex virus*, and *H pylori* in 50 carotid atherosclerotic plaque specimens using polymerase chain reaction and immunocytochemistry techniques. [Hagiwara et al, 2007] The investigators reported that the results of the plaque examination, serum antibodies and the number of seropositive antibodies to these micro-organisms did not correlate with the severely stenotic, ulcerative, or symptomatic plaques. [Hagiwara et al, 2007]

The completion of the L-PEPS study provided a unique opportunity to investigate for the first time, in a case-control fashion, the infectious burden of the 3 most common atypical respiratory pathogens in elderly stroke / TIA patients. Although the data were suggestive of an association between stroke / TIA and chronic infectious burden of atypical respiratory pathogens as evidenced by IgG, but not IgM, seropositivity, a small sample size was suggested by the wide confidence intervals (Table 4). The association was statistically significant ( $P = 0.04$ ) before adjustment for confounders and close to significance after adjustment ( $P = 0.06$ ). When recurrent stroke / TIA cases were excluded from analysis, the overall association became statistically less

significant, with  $P=0.09$  before adjustment, and  $P=0.15$  after adjustment. With only 3 estimates of ORs, the probability value for a test of trend across the ORs was not significant. However, the OR for stroke / TIA and infectious burden after adjustment appeared strongest in subjects infected with at least 3 pathogens: OR= 6.67, 95% CI 1.22 to 37.04, with OR= 7.09, 95% CI 1.05 to 47.62 for first-ever cases of stroke / TIA. Table 5 showed that specific combinations of *C pneumoniae*-*M pneumoniae*, *M pneumoniae*-*L pneumophila*, or *L pneumophila*-*C pneumoniae* did not increase the risk of stroke / TIA significantly. [Ngeh and Goodbourn, 2005]

A number of bacteria and viruses may contribute to atherogenesis and atherothrombosis through inflammatory / immunological mechanisms. [Linsberg and Grau, 2003] Respiratory infections like influenza virus, [Lavalley et al, 2002] *C pneumoniae* [Linsberg and Grau, 2003] and *M pneumoniae* [Ngeh et al, 2004(b)] have been associated with cerebrovascular disease. *C pneumoniae* is the micro-organism most investigated in the ‘infectious hypotheses’ of atherosclerosis. *L pneumophila*, like *C pneumoniae*, is a gram-negative, intracellular bacterium that may exert a chronic immunological response. [Linsberg and Grau, 2003] [Espinola-Klein et al, 2002(a)] [File et al, 1998] [Hindiye and Carroll, 2000] [Stout and Yu, 1997] [Fields et al, 2002] Together, *L pneumophila*, *C pneumoniae* and *M pneumoniae* share similar microbiological and clinical features, including neurological manifestations. [File et al, 1998] [Hindiye and Carroll, 2000] [Johnson et al, 1984] [Easterbrook and Smyth, 1992] [Yucesan and Sriram et al, 2001] They are important atypical respiratory pathogens that cause 8% to 50% of cases of community-acquired pneumonia. [File et al, 1998] [Ngeh and Goodbourn, 2005]

Serological methods such as indirect fluorescence antibody (IFA) and ELISA have test sensitivity of 40 to 80% and specificity of 95 to 99%. [File et al, 1998] [Hindiye and Carroll, 2000] [Murdoch, 2003] They have been recommended for epidemiological studies of *Legionella* infection. [File et al, 1998] [Hindiye and Carroll, 2000] [Murdoch, 2003] The ELISA test is less subjective than the IFA standard reference test. [Murdoch, 2003] [Barka et al, 1986] According to the manufacturer, Vircell ELISA IgM used in the L-PEPS study had sensitivity of 83% and specificity of 97% compared with an immunofluorescence test. The sensitivity and specificity for Vircell ELISA IgG was reported as 95% and 97%, respectively. [Ngeh and Goodbourn, 2005] More recently, the clinical sensitivity and specificity for Vircell ELISA were determined and reported as 92.3% and 100% for the IgM ELISA, and 43.3% and 96.6% for the IgG ELISA. [Diederer et al, 2006] Compared to a validated Serion classic ELISA, the agreement, sensitivity, and specificity were 89.5%, 82.0%, and 97.6% for Vircell IgM ELISA, and 81.9%, 88.9%, and 78.0% for the Vircell IgG ELISA. [Diederer et al, 2006] As the Vircell ELISA kits used in the L-PEPS study were far from perfect, the results in the study have to be read in that context.

The C-PEPS, M-PEPS, and L-PEPS studies had several limitations. The number of controls was small and less than that of cases. The controls were selected from admitted patients and could be biased. The seroprevalences of *C pneumoniae*, *M pneumoniae*, and *L pneumophila* among the medical condition groups of the controls had been analysed. (Table 6). There was no association between atypical respiratory infection and medical conditions in the controls. Although the result was statistically significant for *M pneumoniae* IgM (P= 0.02), the number of positive controls was so

small (n= 9) that this could be due to chance. Silent or subclinical cerebrovascular disease in the controls could not be excluded, although CT would have been helpful to detect asymptomatic stroke in this group. Subclinical atypical respiratory infection or even epidemic during the study period might induce an antibody response in the controls, thereby obscuring any genuine serological association between infection and stroke / TIA.

Patients with stroke could be more susceptible to respiratory tract infection. It could be argued that infection may not be a cause of the present stroke event but a result of past / recurrent stroke. However, the results in Table 7 showed that the seroprevalences of the pathogens did not differ between cases with first-ever or recurrent stroke / TIA.

## **5.5. Conclusions**

Although a significant association between individual serological markers of *C pneumoniae*, [Ngeh et al, 2003] *M pneumoniae*, [Ngeh et al, 2004(b)] *L pneumophila*, and acute cerebrovascular events was not apparent, this preliminary case-control study nevertheless suggested that a collective burden of chronic atypical respiratory infection (IgG seropositivity) may be associated with an increased risk of acute stroke / TIA. Whether seroprevalences of atypical respiratory pathogens in elderly patients could reflect a life-long recurrent infection or an infective burden related to an aetiological link with atherosclerosis will need to be confirmed in larger, prospective outcome studies.

The next chapter will present the results of a pilot study on the seroprevalence of *Coxiella burnetii*, another atypical respiratory pathogen, in the same cohort of patients as in the C-PEPS study.



## **Chapter 6**

***Coxiella burnetii* in elderly patient with stroke (C-BEPS): a case-control study on the seroprevalence of *Coxiella burnetii* in elderly patients with acute stroke / transient ischaemic attack**

## 6.1. Introduction

Historically, *Rickettsiae* were the microorganisms used in 1889 to successfully induce fatty sclerotic changes after inflicting slight mechanical injury to the arterial wall of the aorta of a rabbit. [Gilbert and Lion, 1889] [Lovey et al, 1999] As a member of the *Rickettsiaceae* family, *Coxiella burnetii* causes Q fever, a zoonosis with a worldwide distribution with the exception of New Zealand. [Maurin and Raoult, 1999] It is well known that chronic *C. burnetii* infection commonly causes endocarditis and less commonly causes vascular infections of aneurysms and vascular grafts. [Maurin and Raoult, 1999] [Lovey et al, 1999] *Coxiella burnetii* is also a well recognised atypical respiratory pathogen. [Maurin and Raoult, 1999] [Ngeh and Goodbourn 2005(b)] In Nova Scotia, Canada, *Coxiella burnetii* was the third most frequently recognised bacterial aetiological agent of atypical pneumoniae, after *Mycoplasma pneumoniae* and *Chlamydia pneumoniae*; and it was more frequently diagnosed than psittacosis and legionellosis. [Marrie et al, 1996] [Maurin and Raoult, 1999]

The seroprevalences of three common atypical respiratory pathogens: *Chlamydia pneumoniae*, *Mycoplasma pneumoniae* and *Legionella pneumophila*, in case-control studies involving an elderly cohort of stroke / transient ischaemic attack (TIA) and medical patients were published and reported in previous chapters of this thesis. It was concluded that the risk of stroke / TIA appeared to associate with the aggregate number of chronic infectious burden of these atypical respiratory pathogens. [Ngeh and Goodbourn, 2005(a)] Conceivably, other respiratory pathogens may also contribute to this association. The aim of this ‘*Coxiella burnetii* in elderly patients with stroke’ or ‘C-BEPS’ study was to determine the seroprevalence

(immunoglobulins, IgG and IgM) of *Coxiella burnetii* in the same cohort of patients as in the original ‘*Chlamydia pneumoniae* in elderly patients with stroke’ or ‘C-PEPS’ study. [Ngeh and Goodbourn, 2005(b)]

## **6.2. Subjects and Methods**

The details of the C-PEPS study, from which the present C-BEPS study was based, were described in chapter 2 of this thesis. In keeping with the ‘*Legionella pneumophila* in elderly patients with stroke’ or L-PEPS study discussed in the previous chapter, the C-BEPS study also excluded the 7 cases of haemorrhagic stroke without acute infarction as detected on computed tomography (CT) head scan. Using commercial enzyme-linked immunosorbent assay (ELISA) kits (PANBIO Limited, Australia), the seroprevalence of *C burnetii* in 85 ischaemic stroke / TIA patients and 84 medical control patients was determined by laboratory technicians blinded to the case or control status of patients’ serum samples. The C-BEPS study had fewer patients than the C-PEPS study as it had excluded another 8 cases and 3 controls because of a shortage of ELISA kits. [Ngeh and Goodbourn, 2005(b)]

## **6.3. Results**

The seropositivity of *C burnetii* IgG was found in 2 (2.4%) out of the 85 ischaemic stroke / TIA patients, and none in the 84 control medical patients. None of the 85 stroke / TIA patients and 3 (3.6%) of the 84 control medical patients were seropositive for *C burnetii* IgM. [Ngeh and Goodbourn, 2005(b)] The 2 stroke patients tested seropositive for *C burnetii* IgG were both females aged 70 years and 93

years. Whereas the 3 control medical patients tested seropositive for *C burnetii* IgM were also all females aged 75 years, 78 years and 91 years.

#### **6.4. Discussion**

Previous studies in the United Kingdom using immunofluorescence test reported a *C burnetii* IgG seroprevalence of 15% to 27% in farm workers, and 4% to 11% in people working in non-farming sectors. [Maurin and Raoult, 1999] [Thomas et al, 1995] [Davies et al, 1997] Worldwide, *C burnetii* seroprevalences in different human populations, not during the outbreaks, have been reported to range from less than 1.5% [Kim et al, 2006] to over 50% in hyperendemic areas [Ruiz-Beltran et al, 1990] [Pascual-Velasco et al, 1998]. [Maurin and Raoult, 1999] The *C burnetii* seropositivity of 2.4% for IgG and 3.6% for IgM in the C-BEPS study is within the range of *C burnetii* seroprevalences published internationally.

Some studies reported no association between *C burnetii* seropositivity and age [Thomas et al, 1995] or sex, [Davies et al, 1997] [Tissot Dupont et al, 1992] and in subjects older than 64 years [Suarez-Estrada et al, 1996]. However, several Spanish studies have reported *C burnetii* seroprevalence to be significantly higher in males than females, [Sanzo et al, 1993] and to increase significantly with age [Ruiz-Beltran et al, 1990] [Sanzo et al, 1993] [Marrie and Pollak, 1995] to over 70% in those over the age of 65 years. [Pascual-Velasco et al, 1998] In Taiwan, the seroprevalence of *C burnetii* was 4.2% in 616 subjects without any signs compatible with acute Q fever; and the antibody prevalence rate was higher in males than in females, and peaked in persons aged 61 to 70 years. [Ko et al, 2000] In a recent Spanish study, *C burnetii*

seroprevalence in 216 subjects was estimated to range from 9% to 15%; and the seropositive cases were significantly higher in patients over 44 years of age. [Cardenosa et al, 2006] However, the data in this Spanish study showed a sex ratio of 1:1, [Cardenosa et al, 2006] which was similar to that reported in another study in Nova Scotia [Marrie and Pollak, 1995].

The median age of the elderly patients in the C-BEPS study was 80 years. The relatively low *C burnetii* seropositivity of 2.4% to 3.6% found in the C-BEPS study would suggest that *C burnetii* exposure was rare and that seroprevalence did not increase with age in this cohort of elderly patients living in the urban areas of north-east London, England. This would be in keeping with the other two British studies that showed no association between *C burnetii* seropositivity and age. [Thomas et al, 1995] [Davies et al, 1997] Interestingly, all the 5 elderly patients tested seropositive in the C-BEPS study were females. Although this could be due to chance, the finding was in contrast to most studies which showed that *C burnetii* seropositivity was either increased in the males or not associated with sex.

A limitation of this C-BEPS study was that a more detailed occupational / farming history of the patients was not possible to obtain retrospectively. However, in one study, 90% of *C burnetii* seropositive blood donors reported no contact with farm animals, and pet ownership was reported to have no impact on the seroprevalence. [Bartolome et al, 2007] Moreover, as *C burnetii* can be transported by the wind, a substantial number of infected patients have also reported no direct contact with animals. [Tissot-Dupont et al, 1999] It was noted that there was no clinical evidence of acute or serious *C burnetii* infection especially in the control medical patients

recruited in the C-BEPS study. This could be explained by subclinical or asymptomatic infection which could occur in almost 60% of Q fever cases, [Maurin and Raoult, 1999] [Marrie and Raoult, 2002] or related to a problem with ELISA test specificity [Ngeh and Goodbourn, 2005(b)].

Detectable levels of specific IgG or IgM antibodies are considered evidence of past or recent *C burnetii* infection, respectively. (Cat No. E-QFB01G and E-QFB01M, PANBIO Limited, Australia) According to the manufacturer's data, *C burnetii* ELISA (PANBIO Limited, Australia) had a sensitivity, specificity and agreement of 72.2%, 100%, and 79.6%, respectively, for IgG, and 97.1%, 84.4%, and 89.8%, respectively, for IgM, when tested against the immunofluorescence reference method. Others reported that ELISA for the diagnosis of acute Q fever had sensitivity of 80% and sensitivity of > 99% for *C burnetii* anti-phase II IgG ( $\geq 1:1,024$ ), and sensitivity of 84% for *C burnetii* anti-phase II IgM ( $\geq 1:512$ ). [Waag et al, 1995] [Fournier et al, 1998] These results were not perfect. However, others have demonstrated that ELISA technique was even more sensitive than the immunofluorescence assay and could thus be used for the serodiagnosis of Q fever. [Peter et al, 1988] [Cowley et al, 1992] [Fournier et al, 1998] Indeed, ELISA had been proposed and recognised as a useful tool for seroepidemiological studies, [Peter et al, 1987] [Maurin and Raoult, 1999] [Kovacova and Kazar, 2002] and for the diagnosis of both acute and chronic Q fever [Kovacova and Kazar, 2002].

As discussed in previous chapters, various micro-organisms may contribute to atherogenesis and atherothrombosis through inflammatory / immunological mechanisms. Among these micro-organisms, *C pneumoniae* is the one most

investigated and implicated in the infectious hypothesis of atherosclerosis- an acknowledged inflammatory disease. [Ngeh and Gupta, 2004] Like *C pneumoniae*, *C burnetii* is a gram-negative, intracellular bacterium that may result in chronic infection and exert a chronic immunological or inflammatory response. [Maurin and Raoult, 1999] [Lovey et al, 1999] [Marrie and Raoult, 2002] [Bernit et al, 2002] [Ngeh and Gupta, 2004] [Ngeh and Godbourn, 2005(a)] Furthermore, *C burnetii*, *C pneumoniae*, *M pneumoniae*, and *L pneumophila* are all atypical respiratory pathogens that share similar microbiological and clinical features, including vascular invasion and neurological manifestations. [Ngeh and Godbourn, 2005(a)] [Maurin and Raoult, 1999] [Marrie and Raoult, 2002] [Bernit et al, 2002]

Indeed, *C burnetii* infection was reported to associate with cerebrovascular and ischaemic heart diseases. [Lovey et al, 1999] [Ngeh and Goodbourn, 2005(b)] In a cohort study in Switzerland, people affected by the largest reported outbreak of Q fever in 1983 were followed up 12 years later to evaluate the long term complications of *C burnetii* infection. [Lovey et al, 1999] The investigators reported that the 12-year risk of arterial disease was significantly higher among those patients who had been acutely infected (7%) as compared with those who had never been infected (4%) [Relative Risk (RR)= 2.2; 95% Confidence Interval (CI)= 1.4 to 3.6]. Specifically, there was an increased risk of developing a cerebrovascular event (RR= 3.7; CI= 1.6 to 8.4) and cardiac ischaemia (RR= 1.9; CI= 1.04 to 3.4). The 12 year mortality was significantly higher among the 411 people who had been acutely infected in 1983 (9.7%) (age adjusted RR= 1.8; CI= 1.2 to 2.6) when compared with the 1247 participants who had remained serologically negative in 1983 (7.0%). [Lovey et al, 1999]

In the present C-BEPS study, because of such sparse data and zero counts (i.e. 0/84 and 0/85), it was not possible to estimate any meaningful relative risk, and therefore, impossible to make any conclusion about any association between *C burnetii* infection and stroke / TIA. [Ngeh and Goodbourn, 2005(b)] Nevertheless, the C-BEPS study served as a pilot seroepidemiological survey of *C burnetii* infection in a cohort of hospitalised elderly stroke, TIA and medical patients living in the urban areas of north-east London. The low *C burnetii* seroprevalence found in the C-BEPS study would suggest that *C burnetii* infection was relatively uncommon in the elderly population of north-east London in England.

The next chapter will discuss the prevalence of electrocardiographic rhythms and ischaemic changes in the same cohort of acute stroke / TIA and medical patients as recruited in the C-PEPS study.



## **Chapter 7**

**A case-control study on the prevalence of electrocardiographic rhythms and ischaemic changes in elderly patients with acute stroke / transient ischaemic attack**

## 7.1. Introduction

Electrocardiographic (ECG) abnormalities such as arrhythmias and ischaemic changes are known to occur in acute cerebrovascular disease. [Khechinashvili and Asplund, 2002] [Oppenheimer, 2002] These abnormalities may represent pre-existing coronary heart disease or direct consequences of acute cerebral insults from subarachnoid haemorrhage, ischaemic stroke, or intra-cerebral haemorrhage. [Khechinashvili and Asplund, 2002] [Oppenheimer, 2002] Although cerebrovascular and ischaemic heart diseases (IHD) are associated with ageing, data on the prevalence of ECG changes in elderly patients with acute stroke are limited. [Khechinashvili and Asplund, 2002] This case-control study investigated the prevalence of ECG rhythms and ischaemic changes in elderly patients admitted with acute stroke or transient ischaemic attack (TIA) to a district general hospital in London, United Kingdom. [Ngeh, 2004]

## 7.2. Methods

The data used in this study originated from the '*Chlamydia pneumoniae* in elderly patients with stroke' or 'C-PEPS' study as discussed in chapter 2 of this thesis. Ninety-seven consecutive stroke / TIA and 70 medical control patients who had a 12-lead ECG tracing recorded within 48 hours of their acute admissions in the C-PEPS study were included in this study. [Ngeh, 2004] The control patients who had never had a stroke and who were admitted with acute non-cardiopulmonary disorders were recruited concurrently with the stroke / TIA patients. [Ngeh, 2004]

The patients' ECG recordings were examined for rhythms, and ischaemic changes defined [De Bacquer et al, 2000] as the presence of abnormal Q-wave patterns, ST segment depression or elevation, T-wave inversion or flattening, and left bundle branch block. The prevalence of ischaemic ECG changes in the stroke / TIA patients were compared with that in the control patients using  $\chi^2$  test. Using a logistic regression statistical model to adjust for possible confounding effect of background history of IHD, the odds ratio (OR) of having ischaemic ECG changes in acute stroke / TIA patients was determined. [Ngeh, 2004]

### **7.3. Results**

The median age of all patients was 80 years (range= 65 to 98 years in stroke / TIA patients, 65 to 95 years in control medical patients). Fifty-seven stroke / TIA patients (59%) and 47 control medical patients (67%) were females. Forty-eight stroke / TIA patients (49.5%) and 28 control medical patients (40%) had a history of hypertension, 58 stroke / TIA patients (60%) and 48 control medical patients (69%) had a history of smoking, and 8 stroke / TIA patients (8%) and 7 control medical patients (10%) had a history of diabetes mellitus. [Ngeh, 2004]

Among the 97 acute stroke / TIA patients (87 acute stroke, 10 TIA), 62 were admitted with their first-ever presentation of an acute stroke or TIA. Thirty-four patients had a history of one or more episodes of stroke or TIA. No data for previous stroke or TIA were available for one patient. [Ngeh, 2004]

Electrocardiographic sinus rhythm was observed in 67 stroke / TIA patients (69%) and 47 control medical patients (67%). These included 2 stroke patients in sinus bradycardia and 2 control medical patients in sinus tachycardia. The ECG recordings of 26 stroke / TIA patients (27%) and 17 control medical patients (24%) were in atrial fibrillation. Other rhythms observed were first-degree heart block (in two stroke / TIA patients and two control medical patients), junctional rhythm (one stroke patient and two control medical patients), paced rhythm (one stroke patient), bigeminy (one control medical patient), and first-degree heart block with bigeminy (one control medical patient). [Ngeh, 2004] [Ngeh, 2000]

Ischaemic ECG changes were observed in 54 stroke / TIA patients (56%) and 32 control patients (46%) [Odds ratio (OR)= 1.52; 95% confidence interval (CI)= 0.82 to 2.83; P= 0.18]. In contrast, only 17 stroke / TIA patients (18%) versus 19 control patients (27%) had a history of IHD. Using a logistic regression statistical model to adjust for history of IHD, the OR of having an ischaemic ECG in relation to a stroke / TIA was 1.80 (95% CI= 0.93 to 3.45; P= 0.079). [Ngeh, 2004]

Computerised tomography (CT) head scan results were available for 77 of the 97 stroke / TIA patients. Of the 77 stroke / TIA patients with a CT head scan, 47 were found to have an infarct, 6 had a haemorrhage, 2 had a haemorrhagic infarct, 2 had a haemorrhage with old infarct, and no stroke lesion was demonstrated in 20 patients. The results of these CT head scans have been reported in the C-PEPS study and in chapter 2 of this thesis. [Ngeh et al, 2003] [Ngeh, 2004]

For the 6 stroke patients with only a haemorrhage on CT head scan, 2 of 6 patients (33%) showed atrial fibrillation and ischaemic changes on the ECG. Both ECG recordings of the 2 patients with haemorrhagic infarct showed sinus rhythm, one of which showed ischaemic changes. For the 2 patients whose CT revealed haemorrhage with old infarct, both ECG tracings showed sinus rhythm and ischaemic changes. Thus, for all the 10 stroke patients with haemorrhage on CT head scan, the ECG recordings showed that 2 out of 10 patients (20%) were in atrial fibrillation with the rest in sinus rhythm; and five out of the 10 (50%) showed ischaemic changes. [Ngeh, 2004]

#### **7.4. Discussion**

The vast majority of previous studies on ECG changes in stroke were uncontrolled observational studies on younger patients that focused on ECG changes in the context of subarachnoid haemorrhage. [Khechinashvili and Asplund, 2002] [Davis et al, 1993] This case-control study was the first that investigated the prevalence of ECG rhythms and ischaemic changes prospectively in elderly patients admitted with acute stroke or TIA. As the requests for 12-lead ECG recordings were made by admitting doctors not involved in the study, only 96 out of 100 of the stroke / TIA patients and 70 out of 87 of the control patients in the C-PEPS study had ECG recordings available. [Ngeh, 2004]

In keeping with a clinical review of electrocardiographic changes associated with acute cerebrovascular disease, [Davis et al, 1993] atrial fibrillation was the most common cardiac arrhythmia found on ECG recordings in acute stroke / TIA patients (27%) and control medical patients (24%) in the present study. Atrial fibrillation

occurred in about a quarter of this very elderly hospital population (median age= 80 years), [Ngeh, 2004] and this was much higher than the previously reported range of 1% to 23% in the clinical review [Davis et al, 1993]. In two previous case-control studies with patients with a mean age of 68 years and 66.4 years, the prevalence of atrial fibrillation in the stroke cases versus controls was 21% versus 2% [Dimant and Grob, 1977] and 14% versus 4% [Goldstein, 1979], respectively. In a general population study, the prevalence of atrial fibrillation for the 65 years to 74 years age group was 2.70% for men and 1.53% for women, and was strongly related to age. [De Bacquer et al, 2000] Others have reported that atrial fibrillation was found in 2% of subjects under the age of 75 years, in 5% of all subjects over 75 years, in 14% over 85 years, and in 27% of patients hospitalised or institutionalized aged over 90 years. [Molaschi et al, 1995] In a study involving 340 patients over the age of 80 years, hospitalised for diseases other than cardiovascular, atrial fibrillation was found in 27.9% of the subjects. [Molaschi et al, 1995] This is similar to that of 27% and 24% found in the elderly (median age= 80 years) stroke / TIA patients and control medical patients, respectively, in the present study.

The prevalence of ischaemic ECG findings, defined as abnormal Q-wave patterns, ST segment depression or elevation, T wave inversion or flattening, and left bundle branch block, in a general population aged 65 years to 74 years was 26.7% for men and 31.3% for women. [De Bacquer et al, 2000] Using the same definition for this present study, ischaemic ECG changes were more commonly observed in the stroke / TIA cases (56%) than in the controls (46%) (OR= 1.52; 95% CI= 0.82 to 2.83; P= 0.18). In comparison, a Finnish cross-sectional epidemiological survey on 488 men and 708 women aged 64 to 97 years reported ischaemic ECG findings in 32.9% (95%

CI= 28.7 to 37.1) of men and 39.3% (95% CI= 35.7 to 43.0) of women; whereas only a minority of them reported typical angina pectoris. [Ahto et al, 1998] However, the high frequency of ischaemic ECG changes found in the stroke / TIA patients was not significantly different from that in the group of age-matched controls in the present study (56% vs 46%; OR= 1.52; 95% CI= 0.82 to 2.83; P= 0.18). This in itself suggested that ischaemic ECG changes were not specifically caused by acute stroke or TIA. [Khechinashvili and Asplund, 2002] [Ngeh, 2004] Indeed, others have concluded that ischaemic-like and repolarisation ECG changes were common in patients with acute ischaemic stroke, and that these changes were more likely to be due to pre-existing heart disease rather than the stroke event. [Familoni et al, 2006]

As ischaemic ECG changes could reflect underlying IHD, the prevalence of IHD was determined from patients' medical history and hospital records. The prevalence of past history of IHD was found to be lower in the stroke / TIA cases (18%) than in the control patients (27%). This unexpected finding suggesting the elderly stroke / TIA population had a lower prevalence of coronary heart disease than age-matched controls remains difficult to explain. It is possible that a history of IHD represents a soft marker of underlying coronary heart disease and is not inclusive of silent cardiac ischaemia. A selective bias in taking the history of IHD could be another possibility, but this is unlikely because the data originally came from the C-PEPS case-control study and histories were collected by the author only. [Ngeh, 2004]

Because the prevalence of IHD was much lower than that of ischaemic ECG changes in both acute stroke / TIA patients (18% vs 56%) and control medical patients (27% vs 46%), ischaemic ECG changes could represent non-specific [Khechinashvili and

Asplund, 2002] findings of silent cardiac ischaemia in both groups. However, compared with the medical controls, ischaemic ECG changes occurred more frequently in acute stroke / TIA patients despite having a lower prevalence of IHD. Using a logistic regression statistical model to adjust for the possible confounding effect of IHD, there emerged a trend of borderline significance towards increased odds ratio for ischaemic ECG changes in acute stroke or TIA (OR= 1.80; 95% CI= 0.93 to 3.45; P= 0.079). [Ngeh, 2004]

The prevalence of atrial fibrillation and ischaemic changes appeared similar in both ischaemic and haemorrhagic strokes, although there were much fewer cases of haemorrhagic stroke in this study. In elderly patients with acute stroke or TIA, ECG changes may well reflect underlying, pre-existing coronary heart disease. This is not surprising because cerebrovascular and ischaemic heart diseases share many risk factors and commonly co-exist in the older population. Indeed, more than a third of stroke patients have asymptomatic coronary heart disease. [Sen and Oppenheimer, 1998] However, a non-specific effect of an acute medical illness on ECG rhythm and ischaemic changes is also a possibility. [Ngeh, 2004]

A direct consequence of a neurological insult from acute cerebrovascular disease on ischaemic ECG changes is well recognized. [Khechinashvili and Asplund, 2002] [Oppenheimer, 2002] [Davis et al, 1993] [Sen and Oppenheimer, 1998] [Oppenheimer and Hachinski, 1992] The proposed mechanisms include an increased sympathetic outflow, catecholamine-mediated cardiac injury and neural-mediated myocytolysis during the acute phase of a stroke. [Khechinashvili and Asplund, 2002] [Oppenheimer, 2002] [Davis et al, 1993] [Sen and Oppenheimer, 1998]



[Oppenheimer and Hachinski, 1992] In addition, involvement of the insular cortex after stroke may be associated with cardiac autonomic deregulation and arrhythmias. [Oppenheimer, 2002] [Sen and Oppenheimer, 1998] [Oppenheimer and Hachinski, 1992] Studies have shown the lateralisation of cardiac autonomic regulation in many species including man, with cardiac sympathetic tone predominantly regulated by right insular regions, and parasympathetic cardiac mechanisms regulated by the left insula. [Oppenheimer, 2002] [Oppenheimer et al, 1992] [Zhang et al, 1998] However, coincidental myocardial infarction in acute stroke has been reported to be as high as 15%. [Davis et al, 1993] Furthermore, the most common cause of death and new morbidity after stroke is from cardiac disease and may be unrelated to the severity of the acute stroke event. [Oppenheimer, 2002] [Wong et al, 2003] In summary, ECG abnormalities associated with stroke may be viewed as due to 3 interacting processes: (a) pre-existing atherosclerosis or hypertensive cardiovascular disease producing left ventricular ischaemic changes, Q waves, arrhythmias, bundle branch blocks, and left ventricular hypertrophy *prior* to the stroke; (b) ischaemic, arrhythmic, and repolarisation changes due to increased sympathetic outflow *during* the stroke; and (c) myocardial necrosis or myocytolysis *precipitated* by (a) or (b) or both. [Goldstein, 1979] [Myers et al, 1982] [Oppenheimer, 2002]

This study had several limitations. Like the C-PEPS study, this study was designed to have a 90% power to detect an OR of  $\geq 3.0$ . While the study might rule out ischaemic ECG changes as a major, specific consequence of an acute cerebrovascular event in this elderly population, a smaller effect that may be significant could not be excluded due to the small sample size. A much larger study would be required to effectively rule out or confirm the significance of ischaemic ECG changes in acute stroke or TIA

in the older population. However, the possibility of beta error in the study should be acknowledged. [Ngeh, 2004]

Prior ECG recordings were not available for comparison with that taken after acute admission of the cases and controls. The true incidence of new ECG changes such as arrhythmia and ischaemic changes after acute stroke or TIA and medical illness could not be determined in this study. However, a previous study has reported that arrhythmias of any type occurred in 41/150 (27%) patients with acute stroke, and new arrhythmias occurred in 13/53 (25%) patients who had prior available ECG tracings for comparison. [Goldstein, 1979] Of 13 patients with cerebral embolic stroke who had prior available ECG tracings, 8 (62%) showed atrial fibrillation on the current ECG, and in 4 of these 8 patients (50%), atrial fibrillation was a new finding. [Goldstein, 1979] Another study has reported ECG findings that revealed a new diagnosis of atrial fibrillation in 28 out of the 1200 (2.3%) patients with TIA who has no prior history of atrial fibrillation. [Elkins et al, 2002]

Electrocardiographic ST depression or T wave inversion has been reported to occur in 59/150 (39%) of patients with acute stroke, and new ischaemic changes in 11/53 (21%) of patients who had prior available ECG tracings. [Goldstein, 1979] Another study has reported that patients with acute ischaemic stroke or TIA had a 29% prevalence of ST segment depression within the first five days after their event, as detected by continuous ECG monitoring. [McDermott et al, 1994] In logistic regression analysis, increasing age ( $P < 0.02$ ) and a left-sided neurological event ( $P < 0.01$ ) were significant predictors of ST segment depression; whereas atherosclerotic risk factors, history of cardiac disease, and mean arterial pressure were not. [McDermott et al, 1994] In

another study involving stroke patients (mean age=  $73 \pm 9.3$ ) without primary heart disease, new ST-T ECG changes of a small magnitude were seen in about half of the cases (13/27 patients) with daily ECG recordings, during the first 10 days after the acute stroke event. [Lindgren et al, 1994]

Compared with continuous ECG monitoring and serial follow-up ECG recordings, single 12-lead ECG recordings in the present study were likely to underestimate the true incidence of ECG abnormalities after acute stroke and medical admissions. A study involving 271 stroke patients has reported that the incidence of detected arrhythmia was 61% with ECG monitoring and 41% in the unmonitored group. [Reinstein et al, 1972] [Davis et al, 1993] Although ECG monitoring resulted in increased arrhythmia detection, this was reported to have little or no effect on 30-day mortality. [Reinstein et al, 1972] [Davis et al, 1993] However, others reported that ECG dysrhythmia e.g. atrial fibrillation, is one of the most important indicators of 30-day mortality in patients with first-time ischaemic stroke (OR=5.2; 95% CI= 2.37 to 13.77). [Szcudlik et al, 2000] Furthermore, 3-month mortality in 692 patients with ischaemic stroke was predicted by atrial fibrillation (OR= 2.0; 95% CI= 1.3 to 3.1), and ischaemic changes such as ST elevation (OR= 2.8; 95% CI= 1.3 to 6.3), ST depression (OR= 2.5; 95% CI= 1.5 to 4.3), and inverted T wave (OR= 2.7; 95% CI= 1.6 to 4.6); independent of stroke severity, pre-stroke disability and age. [Christensen et al, 2005] However, none of the ECG changes reached significance in 223 patients with TIA. [Christensen et al, 2005] A recent study has reported that Holter 24-hour ECG recording detected atrial fibrillation in 5% of acute ischaemic stroke or TIA patients with a normal standard ECG (7/139 patients), whereas an event loop recorder i.e. a 7-day ambulatory ECG monitoring detected atrial fibrillation in 5.7% of patients

with a normal standard ECG and Holter (5/88 patients). [Jabaudon et al, 2004] The investigators concluded that an event loop recorder should be considered in every acute ischaemic stroke or TIA patient in whom a cardioembolic mechanism was suspected. [Jabaudon et al, 2004] Indeed, previous studies showed that atrial fibrillation in stroke patients predicted poor outcome at 1 month and 1 year in comparison with subjects in sinus rhythm, [Feinberg et al, 1999] [Lin et al, 1996] with mortality rates of 50% at 1 year. [Carter et al, 2007] In keeping with previous studies, [Vernino et al, 2003] [Marini et al, 2005] atrial fibrillation was recently reported to be a significant and independent predictor of mortality in ischaemic stroke patients followed up for a median of 7.4 years. [Carter et al, 2007]

In addition to conventional risk factors such as older age, male sex, history of angina and diabetes, investigators have reported that ECG evidence of anterior infarction, inverted T wave and left ventricular hypertrophy were all independent predictors for cardiac events in patients with TIA or minor ischaemic stroke. [Pop et al, 1994] Another study has reported that the 90-day risk for a cardiac event was greater in those TIA patients who had any abnormal ECG findings (4.2% vs 0.6%;  $P < 0.001$ ), even after adjustment for medical history and examination findings (OR= 6.9; 95% CI= 1.6 to 29.5;  $P = 0.009$ ). [Elkins et al, 2002] However, the ECG abnormalities were not found to associate with risk for stroke or death. [Elkins et al, 2002] In another study, the 6-month mortality rate in the acute ischaemic stroke patients was significantly higher in patients with abnormal ECG changes when compared to those patients with normal ECG changes (38.9% versus 15.2%;  $P = 0.018$ ). [Bozluolcay et al, 2003] Specific ECG abnormality such as long QT<sub>c</sub> interval (e.g. in lead V6) or increased QT dispersion which may predict future cardiac death in stroke survivors

[Wong et al, 2003] or worse functional outcome [Lazar et al, 2003] [Fure et al, 2006] was not investigated in the present study. Indeed, insular involvement has been reported to independently predict QT prolongation in small infarcts. [Tatschl et al, 2006] However, the main purpose of this present case-control study was to focus on the prevalence of ECG rhythms and ischemic changes in an elderly population within 48 hours of their acute hospital admission. [Ngeh, 2004]

Indeed, many studies have suggested a correlation between abnormal ECG findings and adverse stroke outcomes. [Davis et al, 1993] [Pop et al, 1994] [Dimant and Grob, 1977] [Goldstein, 1979] [Szczudlik et al, 2000] [Elkins et al, 2002] [Wong et al, 2003] Future adequately powered prospective studies may be designed to investigate the relationships between new ECG abnormalities or ischaemic changes and clinical outcomes, in conjunction with specific evaluations of myocardial damage, such as troponin level measurements and echocardiography, in elderly patients presenting acutely with different subtypes of stroke. [Khechinashvili and Asplund, 2002] [Oppenheimer, 2002] [Davis et al, 1993] [Ngeh, 2004] Indeed, a recent study reported that ECG ST depression and Q waves prevalent in acute ischaemic stroke patients were significantly associated with a rise in troponin T, which in turn was significantly associated with a poor short-term neurological outcome (modified Rankin scale > 3) (P= 0.006). [Fure et al, 2006] Others have reported that infarctions in specific brain regions including the right insula were found to associate with elevated serum troponin T level indicative of myocardial injury. [Ay et al, 2006] Recently, a study has reported that raised troponin I was associated with elevation of epinephrine in acute ischaemic stroke, thereby suggesting that activation of the sympathoadrenal system may be an important contributor to myocardial damage. [Barber et al, 2007]

However, the same investigators concluded that an increased troponin I was not associated with insular damage and was not an independent predictor of death or dependency (Rankin > 2). [Barber et al, 2007] For those stroke or TIA patients who have atrial fibrillation, echocardiography may also be used to detect cardiac thrombus, which may be the source of the acute cerebrovascular event. [Ngeh, 2004] Meantime, it seems worthwhile to add ECG information to the conventional risk profile assessment in order to improve the identification, investigation or treatment of subjects at an increased risk for the development of future disease. [De Bacquer and De Backer, 2002]

## **7.5. Conclusions**

This study showed that atrial fibrillation was the most common arrhythmia and accounted for about a quarter of all ECG rhythms in elderly stroke / TIA and medical patients. The high frequency of ischaemic ECG changes found in the stroke / TIA patients (56%) was not significantly different from that in the control patients (46%). Although ischaemic ECG changes could reflect underlying coronary heart disease, they could also be due to non-specific effects of an acute cerebrovascular event or medical illness. After statistical adjustment for a possible confounding effect of IHD, there was a trend of borderline significance to suggest that ischaemic ECG changes were more strongly associated with elderly acute stroke or TIA patients than control medical patients. Larger outcome study will be required to establish and follow-up the significance of ECG abnormalities and ischaemic changes, and also to determine if these changes could represent direct consequences following acute cerebrovascular events in older patients. [Ngeh, 2004]

The next chapter will explore the relationship between socioeconomic deprivation, atypical respiratory infections and survival outcome in the same cohort of patients as in the C-PEPS study.

## **Chapter 8**

**The relationship between socioeconomic deprivation,  
atypical respiratory infections and survival outcome in  
elderly stroke and medical patients**



## 8.1. Introduction

Stroke is primarily a disease affecting older people. Worldwide, stroke remains a significant economic and health burden to an increasingly ageing population. Established risk factors such as hypertension, smoking, diabetes and hyperlipidaemia do not fully account for the clinical and epidemiological occurrences of stroke. Other factors such as socioeconomic deprivation (SED) [Kurth and Berger, 2007] [Cox et al, 2006] [Wong et al, 2006] [Weir et al, 2005] [Arrich et al, 2005] [Aslanyan et al, 2003] [Gillum and Mussolino, 2003] [Kapral et al, 2002] [Jakovljevic et al, 2001] [Peltonen et al, 2000] and infections [Elkind and Cole, 2006] [Grau et al, 2006] have emerged as potentially important modifiable stroke risk factors. However, published data on the relationship between SED, atypical respiratory infections and survival outcome in the context of elderly stroke and control medical patients is very limited. [Ngeh et al, 2007]

The aims of the current study were to investigate in a cohort of elderly stroke / transient ischaemic attack (TIA) and control medical patients simultaneously for: (1) the SED scores of the two groups; (2) the mean SED scores of stroke / TIA and medical control patients who were seropositive or seronegative for atypical respiratory infections; (3) the relationship between SED scores and seropositivity (immunoglobulins IgG and IgM) of individual and aggregate number of atypical respiratory pathogens; (4) the effect of SED on the association between chronic (IgG) atypical respiratory infectious burden and stroke / TIA; and (5) the effect of SED on the survival of stroke / TIA patients and control medical patients. [Ngeh et al, 2007]

## 8.2. Subjects and Methods

The seroprevalences (IgG and IgM antibodies) of atypical respiratory pathogens such as *Chlamydia pneumoniae*, *Mycoplasma pneumoniae*, *Legionella pneumophila* and their association with stroke / TIA versus control medical patients were published and reported in previous chapters of this thesis. The methods of the C-PEPS (*Chlamydia pneumoniae* in Elderly Patients with Stroke), M-PEPS (*Mycoplasma Pneumoniae* in Elderly Patients with Stroke) and L-PEPS (*Legionella Pneumophila* in Elderly Patients with Stroke) case-control studies were also detailed previously in this thesis. [Ngeh et al, 2003] [Ngeh et al, 2004(b)] [Ngeh and Goodbourn, 2005(a)] The original C-PEPS, M-PEPS and L-PEPS studies were approved by the hospital's local research ethics and research and development committees. Informed consent / assent were obtained from patients / carers to access patients' health records as described in the original C-PEPS study. [Ngeh et al, 2003]

The SED and mortality outcome data of the current study were based on the same cohort of patients as in the original C-PEPS study. To ensure homogeneity, 8 acute haemorrhagic stroke cases were excluded. This study focussed on the remaining 92 ischaemic stroke / TIA patients and 87 control medical patients. [Ngeh et al, 2007]

### 8.2.1. Socioeconomic status

Area based measures of socioeconomic status [Cox et al, 2006] such as the English Index of Multiple Deprivation 2004 (IMD 2004) and Income Deprivation Affecting

Older People Index (IDAOPI) were used as indices of SED in the present study. The IMD 2004 and IDAOPI were validated and published in 2004 by the then Office of the Deputy Prime Minister of the United Kingdom (UK) government in collaboration with the Social Disadvantage Research Centre at the Department of Social Policy and Social Research, University of Oxford. [The English Indices of Deprivation, 2004] [Ngeh et al, 2007]

The IMD 2004 and IDAOPI were available to measure SED for the whole of England at a small area level, i.e. a specific, census-based geographical unit known as Super Output Area (SOA), in a consistent manner. [The English Indices of Deprivation, 2004] Each of the 32,482 SOA in England was assigned a deprivation score and rank for the IMD 2004 and IDAOPI which were published officially. [The English Indices of Deprivation, 2004]

All the patients' socioeconomic status in the present study was determined ecologically from their postcode areas of residence as recorded on their recruitment in the original C-PEPS study. These postcodes were also verified by the hospital's computerised administrative record. Each postcode was then entered into the computer to allow for a specific administrative area search for a specific SOA using *Gigateway*, an official web service provided by the Association for Geographic Information, UK. [Gigateway, UK] After obtaining all the patients' resident SOAs, their individual, relative levels of SED were then assigned by the corresponding IMD and IDAOPI numerical scores with rankings. [The English Indices of Deprivation, 2004] [Ngeh et al, 2007] For example, a higher numerical scoring would correspond

to a higher degree of deprivation and a lower ranking number whereby 1 would be the ranking for the most deprived.

### **8.2.2. Survival outcome**

The duration of patients' survival was determined from the date of their hospital admission at the time of recruitment until the date of their death, or if they were still alive, until the end of a 6-year follow-up period ending on the 31 March 2006. The primary end point in the study was all-cause mortality, which was described as an objective, unbiased and clinically relevant outcome. [Shishehbor et al, 2006] In the UK, there is a statutory requirement for an official registration within 5 days of the occurrence of a death. In this study, individual patient's administrative record was cross-checked with the hospital computerised National Health Service (NHS) Strategic Tracing Service [NHS Strategic Tracing Service, 2005] for any record of individual patient's date of death, in May 2006. The NHS Strategic Tracing Service is a database of people, places and NHS organisations in England and Wales, UK. The NHS Strategic Tracing Service receives weekly death data from the UK Office of National Statistics. If there was no statutory public record of a date of death found for a particular patient in this study, it was assumed that the patient was still alive by the 31 March 2006. [Ngeh et al, 2007]

### **8.2.3. Statistical analyses**

The IMD and IDAOPI scores were normally distributed and compared between the groups using the t-test. Survival curves were obtained using Kaplan-Meier plots and

compared using the logrank test. Multiple logistic regressions were used to obtain odds ratios (ORs), with and without adjustment for possible confounding factors. [Ngeh et al, 2007]

### **8.3. Results**

The mean IMD scores of the 92 ischaemic stroke / TIA patients and 87 control medical patients were 23.3 and 25.0, respectively. The difference was calculated as  $23.3 - 25.0 = -1.7$ ; 95% confidence interval (CI)= -4.9 to 1.5;  $P = 0.29$ . The mean IDAOPI score of the stroke / TIA patients and control medical patients were 0.19 and 0.21, respectively. The difference was -0.02; 95% CI= -0.05 to 0.01;  $P = 0.22$ . [Ngeh et al, 2007] The minus signs indicated that the IMD and IDAOPI scores were lower in the cases than controls.

Table 1 shows the difference in the mean IMD and IDAOPI scores between those who were seropositive and seronegative or equivocal in the cohort. Among the control medical patients, there was a statistical difference in the mean IMD and IDAOPI scores ( $P = 0.02$  and  $0.005$ , respectively) between those who were seropositive and seronegative or equivocal for IgM. [Ngeh et al, 2007]

**Table 1.** The difference in the mean IMD or IDAOPI deprivation scores between IgG / IgM seropositive and IgG / IgM seronegative or equivocal patients in stroke / TIA cases and medical control

	Deprivation score	Difference in the mean scores (seropositive minus seronegative or equivocal) and 95% CI	P-value from t-test
IgG			
Cases	IMD	-1.50 (-6.44 to 3.43)	0.55
	IDAOPi	0.002 (-0.046 to 0.051)	0.92
Controls	IMD	-0.22 (-5.10 to 4.66)	0.93
	IDAOPi	-0.014 (-0.062 to 0.034)	0.55
IgM			
Cases	IMD	1.15 (-5.36 to 7.65)	0.73
	IDAOPi	0.033 (-0.031 to 0.096)	0.31
Controls	IMD	7.16 (1.14 to 13.19)	0.02
	IDAOPi	0.084 (0.026 to 0.142)	0.005

IMD indicates Index of Multiple Deprivation

IDAOPi indicates Income Deprivation Affecting Older People Index

The relationship between patients' mean IMD and IDAOPI scores and their IgG and IgM aggregate number of atypical respiratory pathogens is shown in Table 2. There was no clear trend of an association between the number of pathogens and deprivation scores except that for IgM seropositivity, there was a statistically significant association between the number of atypical respiratory pathogens and IDAOPI scores ( $P= 0.004$ ), even after allowing for case-control status. [Ngeh et al, 2007]

**Table 2.** The relationship between patients' mean IMD and IDAOPI deprivation scores and their seropositivity (IgG and IgM) for 0, 1, 2 or 3 of *C pneumoniae*, *M pneumoniae*, and *L pneumophila*

Number of pathogens, IgG	Number of patients	Mean score	IMD	Mean IDAOPI score
0	24	25.7		0.21
1	51	21.7		0.18
2	81	24.6		0.21
3	14	23.9		0.18
All	170*			
P-value		0.37		0.50
P-value after allowing for case-control status		0.44		0.60
Number of pathogens, IgM	Number of patients	Mean score	IMD	Mean IDAOPI score
0	115	22.8		0.18
1	27	26.4		0.24
2	10	24.4		0.20
3	1	44.3		0.49
All	153*			
P-value		0.12		0.003
P-value after allowing for case-control status		0.13		0.004

IMD indicates Index of Multiple Deprivation

IDAOPi indicates Income Deprivation Affecting Older People Index

\*Because 170 was the maximum number of ischaemic stroke / TIA and medical patients tested for *M pneumoniae* IgG and 153 for *L pneumophila* IgM, the numbers do not add up to the total of 179 patients (92 cases and 87 controls)



The unadjusted and adjusted odds ratios (ORs) for stroke / TIA in relation to IgG seropositivity for 0, 1, 2 or 3 of *C pneumoniae*, *M pneumoniae* and *L pneumophila* were published [Ngeh and Goodbourn, 2005(a)] and discussed in chapter 5 of this thesis. To investigate if SED could influence the association between chronic (IgG) atypical respiratory infectious burden and stroke / TIA, the ORs were adjusted for IMD 2004 and IDAOPI scores. As shown in Table 3, the association was significant (P= 0.04) before adjustment, and close to significance after adjusting for IMD (P= 0.052) and IDAOPI (P= 0.054). The association became less significant after adjusting for IMD or IDAOPI, age, sex, hypertension, diabetes mellitus, smoking, ischaemic heart disease and ischaemic ECG (P= 0.09 for IMD, 0.08 for IDAOPI). [Ngeh et al, 2007]

**Table 3.** The effect of IMD and IDAOPI deprivation scores on the odds ratios (ORs) for stroke / TIA in relation to IgG seropositivity for 0, 1, 2 or 3 of *C pneumoniae*, *M pneumoniae*, and *L pneumophila*

Number of pathogens, IgG	No. of cases	No. of controls	Unadjusted	Adjusted for IMD	Adjusted for IDAOPI
0	8	16	1	1	1
1	30	21	2.86 (1.03-7.89)	2.74 (0.98-7.63)	2.78 (1.00-7.69)
2	39	42	1.86 (0.72-4.82)	1.84 (0.71-4.78)	1.86 (0.72-4.85)
3	11	3	7.33 (1.58-33.97)	7.25 (1.56-33.3)	7.14 (1.53-33.3)
All	88	82	P= 0.04	P= 0.052	P= 0.054
Number of pathogens, IgG	No. of cases	No. of Controls	Unadjusted	Adjusted for IMD, age, sex, hypertension, diabetes mellitus, smoking, ischaemic heart disease and ischaemic ECG	Adjusted for IDAOPI, age, sex, hypertension, diabetes mellitus, smoking, ischaemic heart disease and ischaemic ECG
0	8	16	1	1	1
1	30	21	2.86 (1.03-7.89)	3.48 (1.00-12.05)	3.68 (1.07-12.66)
2	39	42	1.86 (0.72-4.82)	1.89 (0.60-5.92)	1.98 (0.64-6.13)
3	11	3	7.33 (1.58-33.97)	6.49 (1.17-35.7)	6.45 (1.17-35.7)
All	88	82	P= 0.04	P= 0.09	P= 0.08
P-value for trend across ORs			P= 0.10	P= 0.23	P= 0.22

IMD indicates Index of Multiple Deprivation

IDAOPi indicates Income Deprivation Affecting Older People Index

The median age of the stroke / TIA patients and control medical patients were 80 years (range= 65 to 98 years) and 82 years (range= 65 to 95 years), respectively, on recruitment. By the end of the 6-year follow-up period, 69 out of 92 stroke / TIA patients (75%) died with a median survival of 22.4 months; 95% CI= 8.7 to 43.8. During the same period, 68 out of 87 control medical patients (78%) also died with a median survival of 36.5 months; 95% CI= 15.4 to 51.7. 14 of the 69 TIA / stroke patients and 6 of the control medical patients in fact died  $\leq 1$  month of their acute hospital admission.

For the cases, the mean IMD for those who died  $\leq 1$  month, died  $> 1$  month, and those who were alive after 6 years were 22.4, 24.6, and 20.4, respectively; and the P-value for the difference between the means was 0.26. The mean IDAOPI for cases who died  $\leq 1$  month, died  $> 1$  month, and those who were alive after 6 years were 0.16, 0.21, and 0.17, respectively; and the P-value for the difference between the means was 0.16. For the controls, the mean IMD for those who died  $\leq 1$  month, died  $> 1$  month, and those who were alive after 6 years were 20.9, 25.2, and 25.6, respectively; and the P-value for the difference between the means was 0.64. The mean IDAOPI for the controls who died  $\leq 1$  month, died  $> 1$  month, and those who were alive after 6 years were 0.14, 0.22, and 0.21, respectively; and the P-value for the difference between the means was 0.24.

Table 4 shows the median survival (in months) of the stroke / TIA patients and control medical patients as divided into tertiles of low, medium and high IMD or IDAOPI scores.

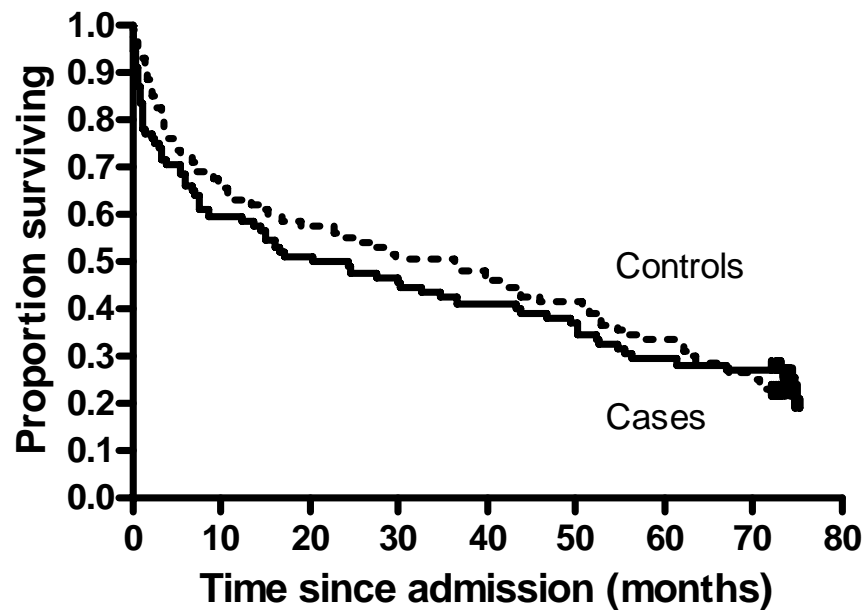
**Table 4.** The median survival (in months) of the stroke / TIA cases and medical controls as divided into 3 tertile groups of low, medium and high IMD or IDAOPI scores

IMD group (tertile)		Median survival in months, 95% CI
Cases	Low ( $\leq 18.29$ )	32.8 (7.6 to 61.4)
	Medium (18.30 to 31.62)	16.5 (5.5 to 50.2)
	High ( $> 31.62$ )	13.6 (6.7 to 43.8)
Controls	Low ( $\leq 18.29$ )	41.6 (10.7 to 61.6)
	Medium (18.30 to 31.62)	16.9 (7.3 to 53.0)
	High ( $> 31.62$ )	29.8 (15.0 to 62.9)
IDAOPI group (tertile)		Median survival in months, 95% CI
Cases	Low ( $\leq 0.14$ )	33.8 (7.6 to 66.9)
	Medium (0.15 to 0.25)	14.1 (5.5 to 24.5)
	High ( $> 0.25$ )	30.1 (6.7 to 52.4)
Controls	Low ( $\leq 0.14$ )	33.4 (7.3 to 61.6)
	Medium (0.15 to 0.25)	41.2 (11.3 to 67.2)
	High ( $> 0.25$ )	22.9 (5.6 to 56.1)

IMD indicates Index of Multiple Deprivation

IDAOPI indicates Income Deprivation Affecting Older People Index

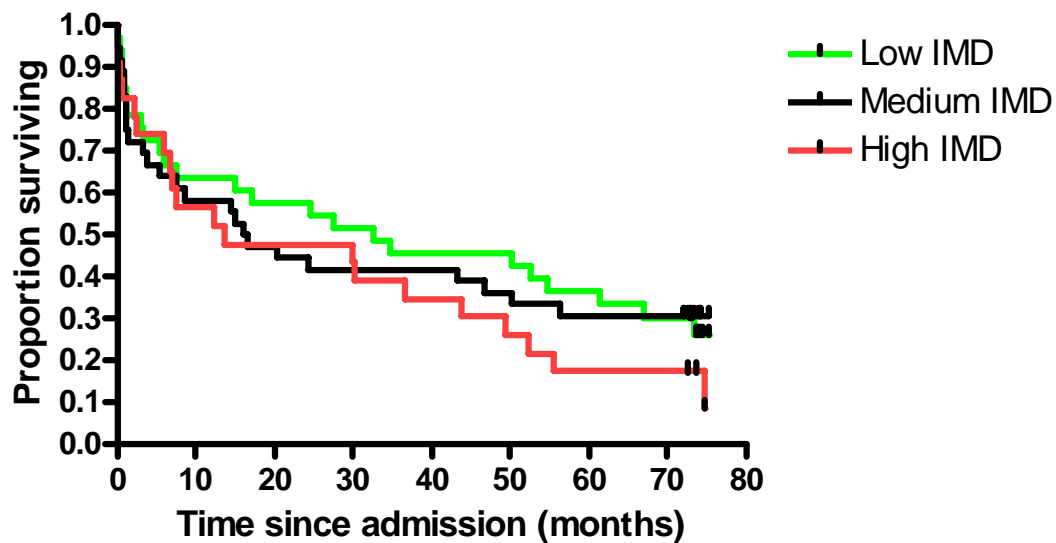
The logrank test for a difference between the Kaplan-Meier survival curves for the stroke / TIA cases and medical controls was not statistically significant;  $P = 0.83$ . (Figure 1a) The hazard ratio of the cases compared to the controls was 1.04; 95% CI= 0.74 to 1.45.



**Figure 1a.** Kaplan-Meier plot for stroke / TIA cases and medical controls

Logrank test,  $P = 0.83$  (no evidence of a difference between the survival curves)

Figures 1b and 1c show the Kaplan-Meier survival curves in stroke / TIA patients and control medical patients using the IMD score. Whereas Figures 1d and 1e show the Kaplan-Meier survival curves in stroke / TIA patients and control medical patients using the IDAOPI score.

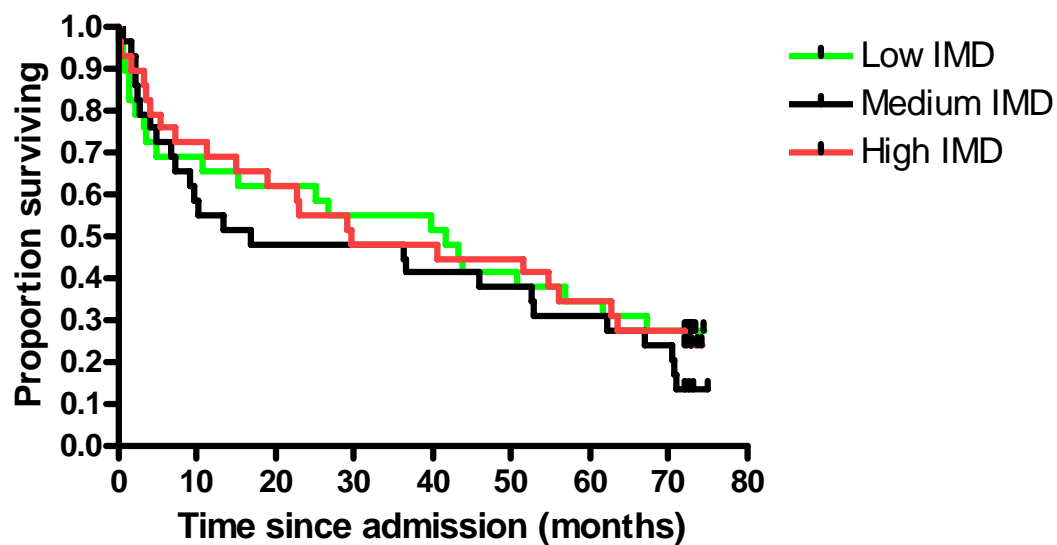


**Figure 1b.** Kaplan-Meier plot for stroke / TIA cases

IMD scores divided into tertiles (based on controls): Low ( $\leq 18.29$ ), Medium (18.30 to 31.62), High ( $> 31.62$ )

Logrank test,  $P = 0.53$  (no evidence of a difference between the survival curves)

Logrank test for trend,  $P = 0.31$  (no evidence of a trend between the curves)

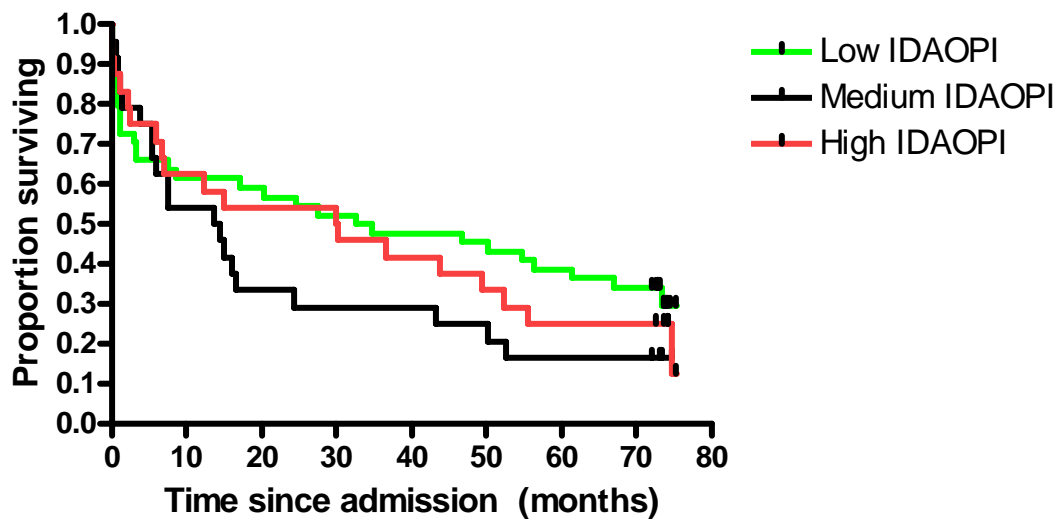


**Figure 1c.** Kaplan-Meier plot for medical controls

IMD scores divided into tertiles (based on controls): Low ( $\leq 18.29$ ), Medium (18.30 to 31.62), High ( $> 31.62$ )

Logrank test,  $P = 0.61$  (no evidence of a difference between the survival curves)

Logrank test for trend,  $P = 0.96$  (no evidence of a trend between the curves)



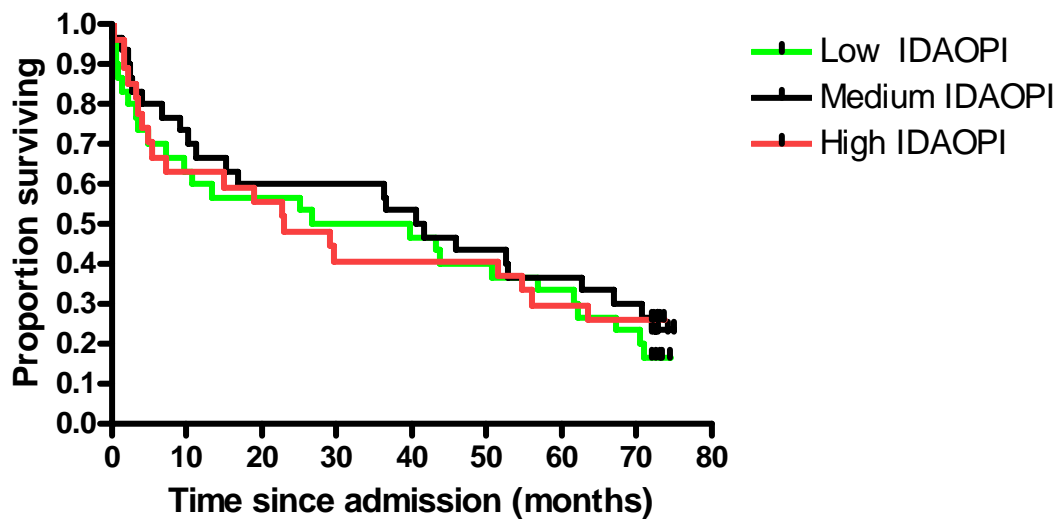
**Figure 1d.** Kaplan-Meier plot for stroke / TIA cases

IDAOPi scores divided into tertiles (based on controls): Low ( $\leq 0.14$ ), Medium (0.15 to 0.25), High ( $> 0.25$ )

Logrank test,  $P = 0.37$  (no evidence of a difference between the survival curves)

Logrank test for trend,  $P = 0.43$  (no evidence of a trend between the curves)





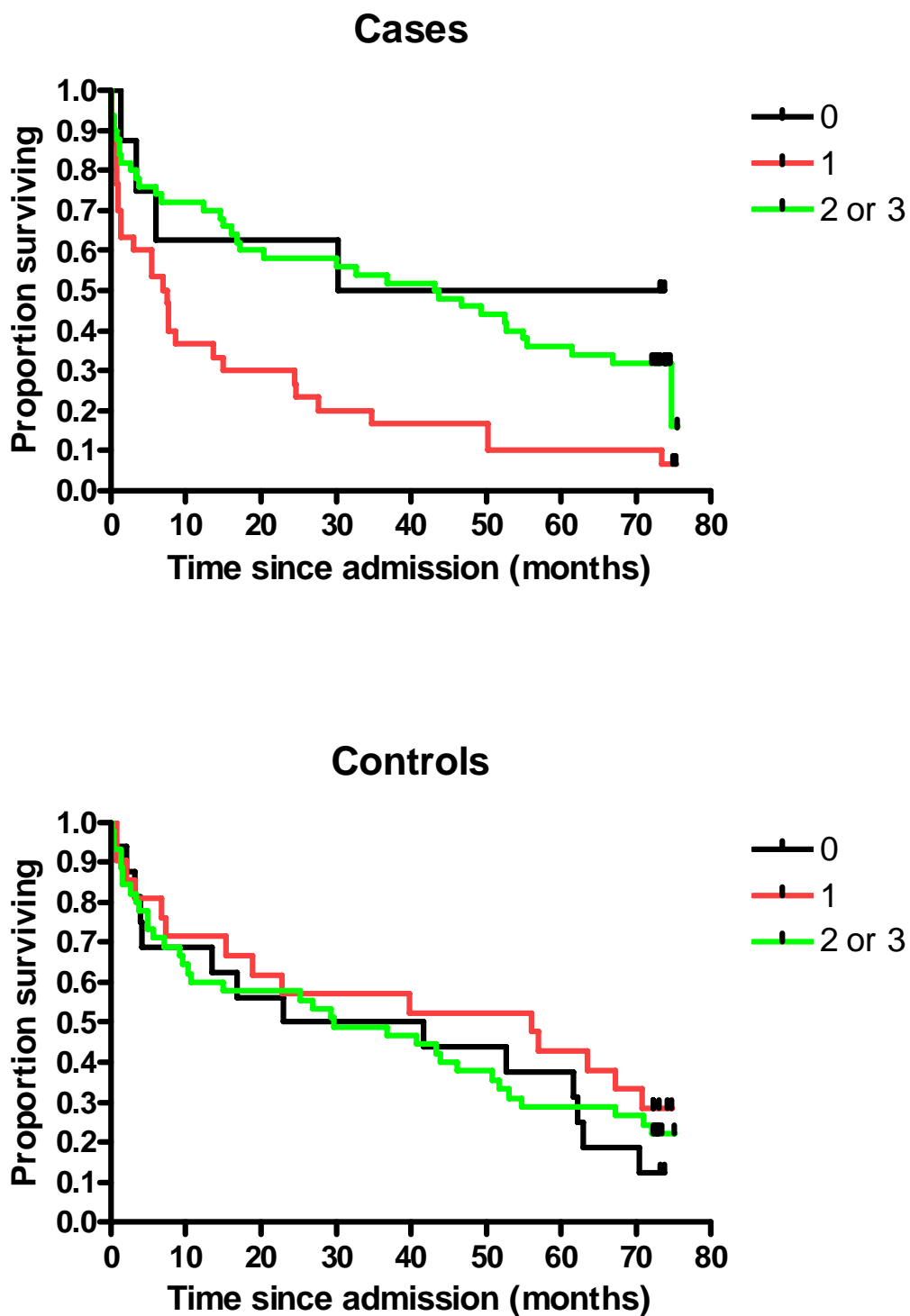
**Figure 1e.** Kaplan-Meier plot for medical controls

IDAOPi score divided into tertiles (based on controls): Low ( $\leq 0.14$ ), Medium (0.15 to 0.25), High ( $> 0.25$ )

Logrank test,  $P = 0.78$  (no evidence of a difference between the survival curves)

Logrank test for trend,  $P = 0.65$  (no evidence of a trend between the curves)

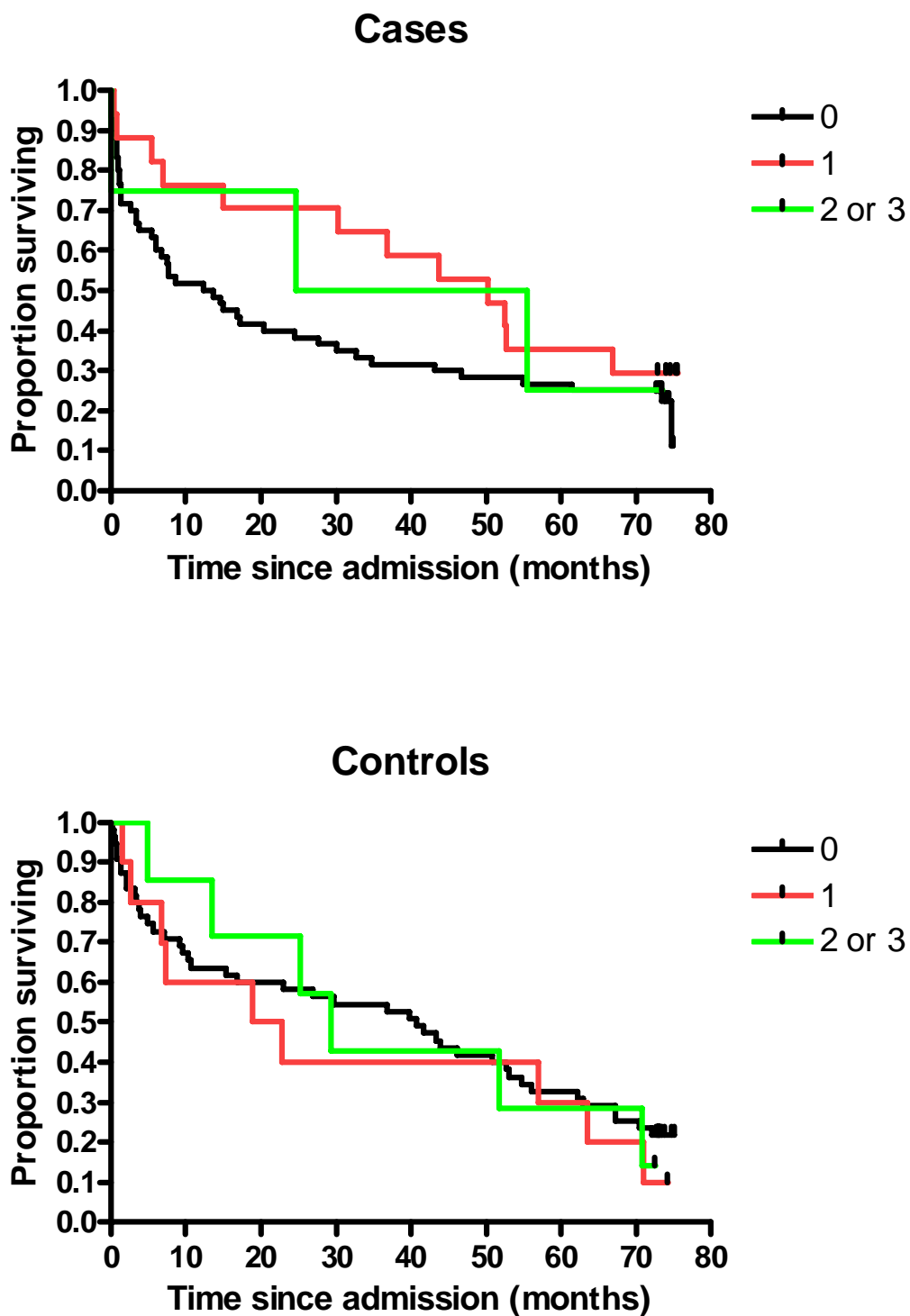
Figures 1f and 1g show the Kaplan-Meier survival curves according to the number of 0, 1, and 2 or 3 of atypical respiratory pathogens in cases and controls, for IgG and IgM seropositivity, respectively



**Figure 1f.** Kaplan-Meier survival curves according to the number of atypical respiratory pathogens for IgG seropositivity

Cases: Logrank test  $P = 0.002$  (Logrank trend test,  $P = 0.34$ )

Controls: Logrank test  $P = 0.59$



**Figure 1g.** Kaplan-Meier survival curves according to the number of atypical respiratory pathogens for IgM seropositivity

Cases: Logrank test  $P=0.36$

Controls: Logrank test  $P=0.84$

## 8.4. Discussion

The term ‘socioeconomic status’ is a framework of concepts and resources ordered in at least 3 levels: material, behavioral and psychosocial. [Kurth and Berger, 2007] For the first time, the English Indices of Deprivation IMD 2004 and IDAOPI were used as an area based measure of socioeconomic status or deprivation in the context of atypical respiratory infections and survival outcome in elderly stroke / TIA and medical patients. The IMD 2004 contained 7 specific domains of deprivation that were weighted in percentage (%) and related to: (1) income (22.5%); (2) employment (22.5%); (3) health and disability (13.5%); (4) education, skills and training (13.5%); (5) housing and services (9.3%); (6) crime (9.3%); and (7) living environment (9.3%). [The English Indices of Deprivation, 2004] The supplementary index, IDAOPI, was specifically created for older people aged over 65 years. [The English Indices of Deprivation, 2004] The denominators of the IMD and IDAOPI were based on the 2001 Census and 2001 Mid-Year Population Estimates. [The English Indices of Deprivation, 2004] Recently, these official English indices of deprivation 2004 were academically peer-reviewed and endorsed. [Alcock, 2007]

Generally, area based measures of socioeconomic status in the UK are consisted of a composite of multiple variables to characterize a geographical area. [Cox et al, 2006] The variables used can vary and are usually taken from census data. [Cox et al, 2006] For example, the Townsend index of deprivation uses unemployment, non-car ownership, non-home ownership, and overcrowding as the variables. [Cox et al, 2006] [Townsend et al, 1987] For the Carstairs deprivation index, the variables used are unemployment, households with no car, overcrowding, and head of household social

class. [Cox et al, 2006] [Carstairs and Morris, 1991] Whereas unemployment, overcrowding, lone pensioners, single parents, ethnic minorities, children under the age of 5 years, head of household social class and 1 year migrants are the variables used in the Jarman deprivation score. [Cox et al, 2006] [Jarman, 1983] [Jarman, 1984] In comparison with these UK indices of SED such as the Townsend, Carstairs and Jarman, the IMD 2004 and IDAOPI were more up-to-date and statistically robust. [The English Indices of Deprivation, 2004]

In this elderly cohort of 92 ischaemic stroke / TIA patients and 87 control medical patients, their socioeconomic status as determined by the difference in their mean IMD ( $P= 0.29$ ) and IDAOPI ( $P= 0.22$ ) scores was not significantly different. [Ngeh et al, 2007]

In the stroke / TIA patients, there was no statistical difference in the mean IMD and IDAOPI scores between those who were seropositive and seronegative / equivocal, for both IgG and IgM of the atypical respiratory pathogens. An association between SED and atypical respiratory infection was not found in the elderly stroke / TIA patients. However, in the medical controls, there was a statistical difference in the mean IMD ( $P= 0.02$ ) and IDAOPI ( $P= 0.005$ ) scores between patients seropositive and seronegative for IgM, but not for IgG. This suggested that an acute atypical respiratory infection could be associated with SED in the elderly medical patients. [Ngeh et al, 2007]

In the present study, an association between the number of atypical respiratory pathogens and deprivation scores was not found in the cohort except that for IgM

seropositivity, there was a statistically significant association between the number of pathogens and IDAOPi scores ( $P= 0.004$ ). This suggested that acute infection of multiple atypical respiratory pathogens or infectious burden was significantly and specifically associated with income deprivation in the elderly, even allowing for case-control status in this study. [Ngeh et al, 2007] In comparison, a recent study reported that although infectious burden by summing of the seropositivity for *C pneumoniae*, *cytomegalovirus*, and *herpes simplex virus 1* was associated with a lower socioeconomic status i.e. employment grade; it did not appear to mediate socioeconomic status differences in cardiovascular disease risk. [Steptoe et al, 2007] However, suggested mechanisms by which socioeconomic status influences cardiovascular disease and stroke risk include differences in major stroke risk factors, in lifestyles and behaviour patterns, in psychosocial factors, in access to health care, and effects of chronic stress mediated by the brain. [Pickering, 1999] [Cox et al, 2006] [Kurth and Berger, 2007]

SED had been positively linked with hospital admission for acute respiratory tract infections and pneumonia ( $P< 0.0001$ ) in all age groups, particularly in children. [Hawker et al, 2003] However, the risk of mortality from respiratory infections was also found to increase with increasing age and deprivation quintile using Townsend deprivation scores. [Jordan et al, 2006] Indeed, highly deprived areas are associated with poverty, and poverty is a predictor of respiratory infections in all age groups. [Rojas, 2007] The mechanisms mediating SED and respiratory infections at individual levels and examination of other areas would merit further studies to identify target groups for effective intervention; although it has been reported that smoking-related causes were found to have the strongest positive relationships with deprivation.

[Romeri et al, 2006] Indeed, behavioural factors such as smoking, drinking, obesity were found to explain a substantial part of the inverse association between socioeconomic status and inflammatory markers e.g. interleukin-6, C-reactive protein, and tumour necrosis factor- $\alpha$ , [Koster et al, 2006] which themselves are biological markers linked to cardiovascular disease. [Pickering, 1999]

As published and discussed in chapter 5 of this thesis, the association between stroke / TIA and IgG seropositivity for 0, 1, 2 or 3 of *C pneumoniae*, *M pneumoniae*, and *L pneumophila* infections was statistically significant before statistical adjustment (P= 0.04). [Ngeh and Goodbourn, 2005(a)] After adjustment for IMD and IDAOPI in this study, the association remained close to significance with P= 0.052 and 0.054, respectively. This suggested that SED on its own had only a modest effect on the association between chronic atypical respiratory infections and elderly stroke / TIA patients. [Ngeh et al, 2007]

The overall association between stroke / TIA and chronic infectious burden of atypical respiratory infection became statistically less significant after adjustment for confounding factors such as age, sex, hypertension, diabetes mellitus, smoking, ischaemic heart disease and ischaemic ECG (P= 0.06). [Ngeh and Goodbourn, 2005(a)] The overall association became even less significant after further adjustment with IMD (P= 0.09) and IDAOPI (P= 0.08) in this study. However, the OR for stroke / TIA in relation to atypical respiratory infectious burden after this further adjustment remained strongest in subjects infected with at least 3 pathogens: OR= 6.5 (CI= 1.17 to 35.7). [Ngeh et al, 2007] Although adjusting SED together with other confounding factors appeared to have weakened the association (P= 0.08 and 0.09) between stroke /



TIA and atypical respiratory infectious burden, it had been criticised as statistical over adjustment in this context. [West, 2000]

In the present study, three quarters of the stroke / TIA patients and control medical patients, respectively, had died by the end of the 6-year follow-up period. Whilst the median age of cases and controls on recruitment in the original C-PEPS study were 80 years, respectively, the median age of death of the stroke / TIA patients and control medical patients were 82 years and 85 years, respectively. There was no statistical difference between the means of IMD and IDAOPi scores for patients who died within one month or after 1 month of their acute admission, or those who remained alive after 6 years of follow-up, in both the cases and controls. Although the median survival of stroke / TIA patients appeared shorter than the control medical patients (22.4 versus 36.5 months), the 95% CI overlapped greatly, indicating that the difference could have been due to chance. There was no survival difference between the groups as divided into tertiles of IMD and IDAOPi deprivation scores, in either the cases or controls. [Ngeh et al, 2007]

Indeed, the relationship between SED and survival outcome after stroke were investigated in several studies with conflicting results. [Cox et al, 2006] [Zhou et al, 2006] [Wong et al, 2006] [Weir et al, 2005] [Arrich et al, 2005] [Aslanyan et al, 2003] [Gillum and Mussolino, 2003] [Kapral et al, 2002] [Jakovljevic et al, 2001] [Peltonen et al, 2000] Some of these studies showed an inverse relationship between higher level of SED and worse survival outcome after a stroke, [Zhou et al, 2006] [Arrich et al, 2005] [Gillum and Mussolino, 2003] [Kapral et al, 2002] [Jakovljevic et al, 2001] whilst others did not demonstrate such a relationship. [Wong et al, 2006] [Weir et al,

2005] [Aslanyan et al, 2003] [Peltonen et al, 2000] For the first time, this 6-year follow-up study suggested that hospitalised elderly patients' duration of survival after an acute stroke / TIA event was not significantly affected by their background SED status on their hospital admission; and that their duration of survival was not statistically different from other hospitalised control medical patients. [Ngeh et al, 2007] In comparison, a study has reported that white, elderly participants (aged 65 years and older) living in the most disadvantaged neighbourhood was associated with higher rates of cardiovascular deaths, even after adjustment for income, education, and occupation (Hazard ratios= 1.5; 95% CI= 1.2 to 1.9). [Diez Roux et al, 2004] However, no neighbourhood differences were observed for non-cardiovascular deaths in this elderly cohort. [Diez Roux et al, 2004] Others have also found no association between poverty area of residence and mortality in older adults. [Diez Roux et al, 2004] [Haan et al, 1987] [Waitzman and Smith, 1998]

Although there were debates about the most appropriate way to detect SED in an elderly population whereby small area-based census variables would generally reflect characteristics of a younger part of the population, it was nevertheless suggested that a combination of SED measures was a very appropriate way to capture and predict the socioeconomic position in an elderly population. [Breeze et al, 2005] [Bowling, 2004] [Grundy and Holt, 2001] Amongst the more conventional SED indicators of education, occupation, income and wealth, it was found that SED of income and wealth were as strongly associated with mortality as, if not more strongly associated with mortality than, education and occupation, even for elderly people aged over 65 years. [Duncan et al, 2002] These suggestions were supportive of the application of IMD and specifically IDAOPI in the current study. [Ngeh et al, 2007] Indeed, income

deprivation has been found to be independently associated with adverse short and long-term mortality among elderly patients following acute myocardial infarction. [Rao et al, 2004]

Apart from area based measures, other specific measures of socioeconomic status such as education, occupation, income, and material ownership do have their own advantages and limitations as reported in a recent review. [Cox et al, 2006] The use of education as a socioeconomic status measure can determine an individual's number of years in full-time education and age on leaving full-time education. An individual's education can be reliably recalled, easily quantified, unaffected by poor health in adulthood, and be a marker of early life circumstances. However, education may be culturally specific, and less applicable to some subgroups or cohorts e.g. the link between education and income is weaker in older women. [Cox et al, 2006]

Occupation as a measure of socioeconomic status is usually a categorical ordering of occupational social class e.g. Registrar General Great Britain social class I – V, [Office of Population Censuses and Surveys, 1991] United States standard occupational classification system, [US Department of Labor, 2000] ISCO-88 international standard classification of occupations [International Labour Office, 1990]. [Cox et al, 2006] It is an advantage that occupation can be an indicator of social status, power, income, and education. However, there are limitations such as subjectivity and inconsistency of ranking systems, limitations in capturing socioeconomic status for some subgroups e.g. women and some ethnic minorities, difficulties incorporating new occupations, people who don't work and older adults, and it may be determined by poor health in adulthood. [Cox et al, 2006]

When income is used as a socioeconomic status measure, it can measure total cash income (month / year), disposable income, individual / household income, and non-cash benefits. An individual's income is a good measure of purchasing capacity e.g. of goods, education, and health care. However, it may be difficult to collect income data, with high non-response rate, and confidentiality concerns. An individual's income may be determined by poor health in adulthood, and it may vary throughout one's life course. Moreover, household members may have unequal access to household income. [Cox et al, 2006]

Material ownership measures ownership of home, car, electrical or white goods e.g. washing machine. Although material ownership is highly correlated with income and education, and is an indicator of standard of living; it may also be an indicator of lifestyle differences. [Cox et al, 2006]

#### **8.4.1. Limitations**

Although the use of area based measures e.g. IMD 2004 and IDAOPI as a measure of socioeconomic status in this study was prone to the bias of 'ecological fallacy' i.e. assuming that all individuals living in an area had similar socioeconomic characteristics, it could nevertheless capture and measure important aspects of SED for a homogeneous elderly population in a small neighbourhood or area (i.e. SOA) that could not be measured individually. [Cox et al, 2006] [Grundy and Holt, 2001] [Kaplan and Keil, 1993]

The SED measures and patients' risk factors in the present study were based on the demographics and clinical information recorded on recruitment. These elderly patients' earlier, life-course, socioeconomic experience was not measured in this study. This could influence the SED measures and risk factors collected at baseline which in turn could change during the follow-up period. [Hart et al, 2000] However, certain vascular risk factors such as hypertension, antihypertensive drug use, prevalence of atrial fibrillation and left ventricular hypertrophy were not found to be associated with SED consistently in a large cohort of elderly Dutch women. [van Rossum et al, 1999] Nevertheless, it has been shown that stroke, stomach cancer and respiratory disease mortality all seem to be related to early life socioeconomic conditions in international ecological studies, [Ben-Shlomo and Davey-Smith, 1991] [Guallar-Castillon et al, 1999] [Leon and Davey-Smith, 2000] and in agreement with findings from prospective studies of individuals. [Davey-Smith et al, 1998] [Davey-Smith et al, 2001] This correspondence in findings between ecological and individual level studies suggests that they reflect underlying causal processes that can be indexed through specific area-based measures with indices relating to social circumstances during early life or through collecting the relevant information on people. [Davey-Smith et al, 2001] Examples of early-life socioeconomic position indicators used in cross-sectional and longitudinal studies included education level, income, occupation, living conditions, family structure and residential mobility. [Chittleborough et al, 2006]

In the present study, it was not possible to determine neither the length of residence nor residential mobility of the elderly patients. [Stjarne et al, 2004] This might lead to a misclassification bias, which could affect the relationship of SED on survival

outcome. [Stjarne et al, 2004] Indeed, it has been suggested that residential environments over the life course, rather than the place of residence in old age, is particularly relevant to longevity. [Diez Roux et al, 2004] Information on patients' long term residence before enrolment in this study was unavailable. The extent to which features of residential environments in old age are correlated with features of places or socioeconomic status earlier in life in this cohort of elderly patients cannot be established. [Diez Roux et al, 2004]

## **8.5. Summary**

In this study, the socioeconomic status of hospitalised acute elderly stroke / TIA patients was not significantly different from the control medical patients. Whilst an association between SED and atypical respiratory infections was not found in the stroke / TIA patients, an association between acute atypical respiratory infection and SED was found to be statistically significant in the control medical patients.

Allowing for case-control status, acute atypical respiratory infectious burden was significantly and specifically associated with income deprivation in this cohort of elderly stroke / TIA and medical patients. However, SED on its own had only a modest effect on the association between chronic (IgG) atypical respiratory infectious burden and elderly stroke / TIA patients.

After 6-years of follow-up, the present study suggested that hospitalised elderly patients' duration of survival after an acute stroke / TIA or medical event was not significantly affected by their background SED status on hospital admission. The

duration of survival of elderly stroke / TIA patients (median= 22.4 months) was not statistically different from that of elderly medical controls (median= 36.5 months). Within either the cases or controls, there was no survival difference between the deprivation groups in tertiles of low, medium and high SED scores.

Socioeconomic deprivation, in particular income deprivation, should be adjusted for in studies on the association between stroke and infections, [Lindsberg and Grau, 2003] especially in the context of acute respiratory infections. Because SED, infections and stroke are potentially important modifiable risk factors, larger, prospective epidemiological and interventional studies to explore the mechanistic relationship between these factors in targeted populations are justified.

The next chapter shall conclude and summarise the main findings of all the published works submitted for this thesis. Some directions for future research study are suggested.

## **Chapter 9**

### **General discussion of published works and conclusions**



## 9.1. Introduction

Stroke as a major clinical manifestation of atherosclerosis, is the leading cause of disability and death in the world. Classical vascular risk factors such as hypertension, smoking, diabetes mellitus and dyslipidaemia do not fully account for the clinical occurrence and epidemiology of stroke. Other novel risk factors such as infection and socioeconomic deprivation have emerged as potential risk factors for stroke.

## 9.2. Infections and stroke

Infections, both acute and chronic, are recognised as potentially important and treatable risk factor for stroke. Many specific acute or recent infections can affect the individual either at a local organ level or systemically. Apart from acute infection of the central nervous system by specific micro-organism resulting in an acute cerebrovascular event, the association between acute systemic infection secondary to acute respiratory tract infection and stroke has also been established.

Since atherosclerosis is recognised as a chronic inflammatory disease, chronic infections are hypothesised to contribute to atherogenesis and atherothrombosis through chronic inflammatory stimuli and immunological mechanisms. These processes could occur in susceptible individuals and synergistically with traditional vascular risk factors. In accordance to the infectious hypothesis of atherosclerosis, specific chronic infections could contribute to atherosclerosis, and thus ischaemic stroke events. Apart from *Cytomegalovirus*, *Helicobacter pylori* and dental pathogens, *Chlamydia pneumoniae* is the micro-organism most studied and implicated in this

context of infection and atherosclerotic vascular diseases such as stroke and coronary heart disease.

### **9.3. *Chlamydia pneumoniae* and atherosclerosis**

*Chlamydia pneumoniae*, an atypical respiratory pathogen, is the micro-organism most investigated in the model of chronic infection and atherosclerotic vascular diseases. The investigations include (1) seroepidemiological observations which are cross-sectional, case-control and prospective in design; (2) laboratory studies which include pathological specimen examinations, animal model experimentations and immunological / molecular studies; and (3) clinical investigations such as human antibiotic interventional trials. Overall, the evidence is compelling but remains inconclusive.

Sero-epidemiological studies have provided weak, circumstantial evidence linking *C pneumoniae* infection and various atherosclerotic vascular diseases. The challenge in this area of study is to identify other (blood) surrogate markers that are accessible and reliable in reflecting underlying endovascular *C pneumoniae* infection, applicable in large scale population studies e.g. antimicrobial treatment studies in the context of stroke. The development of reliable laboratory techniques, and refinement of existing methods e.g. ELISA, to detect blood markers of *C pneumoniae* infection is a priority.

Similarly, the various pathological methods used to detect *C pneumoniae* and its antigens in atherosclerotic specimens will need further improvement and standardisation. Pathological specimen examinations have detected genetic material

of, or even viable, *C pneumoniae* in atheromatous lesions. Although less likely, the possibility of *C pneumoniae* residing in the atheroma as an innocent bystander remains. In conjunction with studies from other areas, pathological specimen studies will help to clarify a possible atherogenic role of *C pneumoniae* infection.

Most animal model studies have demonstrated that *C pneumoniae* could induce atherosclerotic lesions, synergistically or in conjunction with traditional vascular risk factors such as genetic predisposition to develop atherosclerosis, and dyslipidaemia. Animal models will continue to be useful in the investigation of infection induced atherogenic mechanisms, in conjunction with other atherosclerotic risk factors and antibiotic treatment studies.

As a whole, immunological and molecular studies have strongly implicated the roles of *C pneumoniae* in atherogenesis and atherothrombosis. A mechanistic understanding of the interaction between *C pneumoniae* infection and the genesis and progression of atherosclerosis at the molecular or immunological levels will provide possible therapeutic opportunities and/or surrogates of treatment effects. Indeed, *C pneumoniae* has been shown to involve in key events of atherogenesis and atherothrombosis in in-vitro studies.

To date, large-scale clinical antibiotic interventional trials have failed to establish antibiotic treatment benefit in the context of *C pneumoniae* infection in patients with coronary heart disease. However, these studies could not prove or disprove a causal role of *C pneumoniae* in atherogenesis or atherosclerosis, since the process in its early

or stable stages may not always manifest itself clinically as adverse vascular events. Conversely, most of these trials focused on potential antibiotic treatment benefit on patients with pre-existing (advanced) atheromas, and secondary prevention of acute athero-thrombotic events. Future antibiotic treatment studies should incorporate specific surrogate parameters of atherosclerosis, apart from adverse clinical events, as outcome measures. Antibiotic treatment studies should be considered and conducted in the context of other atherosclerotic vascular diseases such as ischaemic stroke / transient ischaemic attack (TIA), in different populations exposed to *C pneumoniae* that may have different atherosclerotic burdens, in both primary and secondary prevention settings. In addition, different antibiotics active against *C pneumoniae* (and against other micro-organisms), and their treatment regimes will need to be developed and standardised in these studies. This is of particular relevance if and when a causal association between *C pneumoniae* and atherosclerosis is proven, since widespread antibiotic misuse and resistance may then become a serious problem. In fact, a minority (4%) of physicians (mostly cardiologists) in the United States have used antibiotics to treat cardiovascular diseases as if it were an infectious disease; although this practice is currently considered inappropriate and premature. [Gimenez-Sanchez et al, 2001]

*Chlamydia pneumoniae* on its own is an important pathogen that causes a wide spectrum of respiratory tract infections and extra-pulmonary diseases worldwide. The recognition of an association between *C pneumoniae* infection and atherosclerotic vascular diseases has generated a great interest in this area of research over the last decade. Although antibiotics active against *C pneumoniae* have failed to show a treatment benefit in the context of coronary heart disease, the body of evidence as a

whole is supportive of *C pneumoniae* as a plausible and modifiable risk factor in atherosclerosis. With the recent discovery of *C pneumoniae*'s genome, the development of an effective vaccination programme in future is becoming a reality that will not only prevent *C pneumoniae* infection but may also clarify if *C pneumoniae* has a causal relationship with atherosclerosis.

#### **9.4. The C-PEPS study**

Seroepidemiological studies have shown a weak relationship between chronic *C pneumoniae* infection and coronary heart disease; although preliminary studies have suggested that the association was stronger for stroke in younger patients. As the occurrence of stroke / TIA increases with age, the relationship between serological markers of *C pneumoniae* infection and older stroke / TIA patients was investigated in the '*Chlamydia pneumoniae* in elderly patients with stroke' or C-PEPS case-control study.

The C-PEPS was a case-control study that investigated the seroprevalence of *C pneumoniae* in elderly stroke / TIA and medical patients. One-hundred white patients aged over 65 years admitted with acute stroke or TIA, and 87 control patients admitted with acute non-cardiopulmonary, non-infective disorders, were recruited prospectively. With the use of SeroCP commercial ELISA kits (Savyon, Israel), the presence of *C pneumoniae* immunoglobulins IgA, IgG, IgM in patients' sera was determined. The seroprevalence of *C pneumoniae* specific IgA, IgG, IgM were 63%, 71%, and 14% in the stroke / TIA group (median age= 80), and 62%, 65%, 17% in the control group (median age= 80), respectively. With the use of a logistic regression

statistical model, adjusting for age and sex, history of hypertension, smoking, diabetes, ischaemic heart disease (IHD), ischaemic electrocardiogram (ECG), the odds ratios (ORs) of having a stroke / TIA in relation to *C pneumoniae* specific IgA, IgG, IgM were 1.04, 1.24, 0.79; and the associated P-values were not significant.

Further analysis of the C-PEPS study identified 43 acute stroke / TIA cases and 44 controls without history of IHD or ischaemic ECG or both. After adjusting for history of hypertension, smoking, diabetes, age and sex, the ORs in this subgroup were 1.40 for IgA (95% confidence interval= 0.53 to 3.65; P= 0.49), 2.41 for IgG (95% CI, 0.90 to 6.46; P= 0.08), 1.55 for IgM (95% CI, 0.45 to 5.40; P= 0.49).

The C-PEPS study concluded that a high seroprevalence of *C pneumoniae* in elderly patients was confirmed, although no significant association between serological markers of *C pneumoniae* infection and acute stroke / TIA events was found. There was, however, a weak trend towards increased ORs for acute stroke / TIA in a subgroup of *C pneumoniae* seropositive elderly patients without any history of IHD or ischaemic ECG. Adequately powered, prospective outcome study may be conducted to see if serological or other blood markers of *C pneumoniae* (endovascular) infection are associated with ischaemic stroke / TIA in younger versus older patients, without clinical evidence of ischaemic heart disease or other atherosclerotic vascular diseases at baseline. The results of such study may help with the targeting of patients for future antimicrobial treatment studies in the context of ischaemic stroke / TIA.

### 9.5. SeroCP ELISA reproducibility

One of the limitations of seroepidemiological studies was the laboratory methods used in serological analyses. The issues included sensitivity, specificity, reproducibility, and operator objectivity of a particular laboratory method or technique. The serological method used in the C-PEPS study was a commercial enzyme-linked immunosorbent assay (ELISA) kit known as SeroCP, which was manufactured by Savyon Diagnostics Limited (Israel). As remarked, ELISA technique was more objective and correlated well with the operator dependent micro-immunofluorescence technique, the reference standard for *C pneumoniae* serological analyses.

With the use of SeroCP ELISA kits, *C pneumoniae* immunoglobulins (Ig) or antibodies in a consecutive series of 122 patients' sera for IgA and IgG respectively, and 138 for IgM, were detected. The ELISA tests on these sera were then repeated. The percentage (%) sample disagreement between the first and repeated ELISA tests were 12%, 16%, and 10% for *C pneumoniae* IgA, IgG, and IgM, respectively. The reproducibility of SeroCP ELISA expressed as Kappa values for IgA, IgG, IgM were 0.73, 0.60, 0.53, respectively ( $P < 0.001$ ). These results suggested that SeroCp ELISA had a good reproducibility for detecting *C pneumoniae* IgA, and moderately good reproducibility for detecting *C pneumoniae* IgG and IgM.

This study has implication in the validity or reproducibility of test results in the C-PEPS and similar seroepidemiological studies. As the results obtained by ELISA is not perfect, they shall have to be read in that context. With further refinement and

improvement, ELISA technique could potentially supercede the immunofluorescence assay and assume an increasingly important role in seroepidemiological studies.

#### **9.6. The M-PEPS study**

*Mycoplasma pneumoniae*, another well known atypical respiratory pathogen, has been linked to coronary heart disease and detected along with *C pneumoniae* in atherosclerotic specimens. Further to the C-PEPS study, there was an opportunity to investigate the serological association between *Mycoplasma pneumoniae* and stroke / TIA. The '*Mycoplasma pneumoniae* in elderly patients with stroke' or M-PEPS case-control study was nested within the C-PEPS study. 95 patients admitted consecutively with acute stroke / TIA, and 82 control patients admitted concurrently with acute non-cardiopulmonary, non-infective disorders, were included in the M-PEPS study.

With the use of SeroMP commercial ELISA kits manufactured by Savyon Diagnostics Limited (Israel), the presence of *M pneumoniae* immunoglobulins IgA, IgG, and IgM in patients' sera was determined. The seroprevalence of *M pneumoniae* IgA, IgG, and IgM in the stroke / TIA patients (median age= 80 years) were 79%, 61%, and 6%, respectively. In the control group (median age= 80 years), the seroprevalence of *M pneumoniae* IgA, IgG, and IgM were 84%, 50%, and 11%, respectively.

With the use of a logistic regression statistical model, adjusting for history of hypertension, smoking, diabetes mellitus, age and sex, history of IHD, and ischaemic ECG, the ORs of having a stroke / TIA in relation to *M pneumoniae* IgA, IgG, and



IgM were 0.63 (95% CI= 0.26 to 1.52; P= 0.31), 1.32 (95% CI= 0.66 to 2.64; P= 0.43), 0.52 (95% CI= 0.14 to 1.92; P= 0.32), respectively. The M-PEPS study showed a high seroprevalence of *M pneumoniae* in an elderly hospital population but ruled out *M pneumoniae* seropositivity as a major risk factor for stroke / TIA in this age group. Adequately powered, prospective outcome study may be warranted to see if serological or other blood markers of *M pneumoniae* infection are associated with ischaemic stroke / TIA or other atherosclerotic vascular diseases in younger versus older patients.

### **9.7. The L-PEPS study**

*Legionella pneumophila* is another well known atypical respiratory pathogen. The ‘*Legionella pneumophila* in elderly patients with stroke’ or L-PEPS was another case-control study based on the same cohort of patients as in the C-PEPS study. Using commercial ELISA kits (VIRCELL SL, Spain), the seroprevalence of *L pneumophila* IgG and IgM antibodies in this cohort of patients were determined. The seroprevalence of *L pneumophila* IgG and IgM were respectively 29% (n= 91) and 12% (n= 81) in the stroke / TIA group, and 22% (n= 86) and 10% (n= 72) in the controls.

With the use of logistic regression to adjust for age, sex, hypertension, smoking, diabetes, IHD and ischaemic ECG, the ORs for stroke / TIA in relation to *L pneumophila* IgG and IgM were 1.52 (95% CI= 0.70 to 3.28; P= 0.29) and 1.49 (95% CI= 0.45 to 4.90; P= 0.51), respectively. The L-PEPS study established the seroprevalence of *L pneumophila* and showed that its seropositivity was not

significantly associated with stroke / TIA, in this cohort of hospitalised elderly patients. Whether serological or other blood markers of *L pneumophila* infection are associated with ischaemic stroke / TIA or other atherosclerotic vascular diseases in younger or susceptible populations may need to be clarified in future studies.

### **9.8. Atypical respiratory infectious burden and stroke**

Previous studies showed that simultaneous or cumulative infections by certain bacteria and viruses, as evidenced by their seropositivities, were associated with cardiovascular events. It was hypothesised that intracellular micro-organisms, such as atypical respiratory pathogens, could exert chronic inflammatory or immunological stimuli contributing to atherosclerosis. Furthermore, atypical respiratory pathogens were known to share similar microbiological and clinical features that included neurological manifestations.

Having established the seroprevalences of *C pneumoniae*, *M pneumoniae* and *L pneumophila* in the C-PEPS, M-PEPS and L-PEPS studies, a further opportunity existed to determine the ORs for stroke / TIA in relation to IgG seropositivity for any 1, 2, or 3 of these chronic atypical respiratory infections. The ORs after statistical adjustment for age, sex, hypertension, diabetes, smoking, IHD, and ischaemic ECG were 3.89 (95% CI= 1.13 to 13.33), 2.00 (95% CI= 0.64 to 6.21), 6.67 (95% CI= 1.22 to 37.04), respectively; P= 0.06.

This further analysis concluded that the risk of stroke / TIA appeared to associate with the aggregate number of chronic infectious burden of atypical respiratory pathogens

such as *C pneumoniae*, *M pneumoniae* and *L pneumophila*. Indeed, the relationship between stroke / TIA and infectious burden appeared strongest in subjects infected with all three atypical respiratory pathogens.

If the infectious hypothesis of atherosclerosis holds true, it is likely that a large but *specific* group of microorganisms (*specific* infectious burden), rather than a selected few, will be discovered to be involved in atherogenesis and atherothrombosis. [Ngeh and Goodbourn, 2005(b)] This concept has implication in the design of future antimicrobial interventional clinical trials in stroke and other atherosclerotic vascular diseases, because subjects infected with a *specific* infectious burden may be identified and randomised to receive *specific* antimicrobial / therapeutic agents or even vaccines. [Ngeh and Goodbourn, 2005(b)] Atypical respiratory pathogens can cause up to 50% of cases of community-acquired pneumonia, [File et al, 1998] in addition to other systemic manifestations. The development of effective vaccination programme in future is becoming a pressing public health issue that will not only prevent atypical respiratory infection but also may clarify if the infectious burden has a causal relationship with stroke and other atherosclerotic vascular diseases. [Ngeh and Goodbourn, 2005(b)]

### **9.9. The C-BEPS study**

The ‘*Coxiella burnetii* in elderly patients with stroke’ or C-BEPS case-control study was based on the same cohort of patients as in the C-PEPS study. The C-BEPS study established the seroprevalence of *Coxiella burnetii*, another well-recognised atypical

respiratory pathogen, in 85 stroke / TIA and 84 control medical patients in hospital setting.

With the use of commercial ELISA kits (PANBIO, Australia), the seropositivity of *C burnetii* IgG was found in 2 out of 85 ischaemic stroke patients (2.4%), and none in the 84 control patients. None of the 85 cases and 3 out of the 84 control patients (3.6%) were seropositive for *C burnetii* IgM. With such sparse data and zero counts (i.e. 0/84 and 0/85), the association between *Coxiella burnetii* infection and stroke / TIA was undetermined. However, the C-BEPS study served as a pilot seroepidemiological survey of *C burnetii* infection in a cohort of hospitalised, elderly stroke / TIA and medical patients from the urban areas of north-east London, and in a non-outbreak setting.

#### **9.10. ECG and stroke**

Electrocardiographic (ECG) abnormalities have been observed in acute cerebrovascular events. An opportunity existed for a case-control study on the prevalence of ECG rhythms and ischaemic changes of 97 elderly stroke / TIA patients and 70 control medical patients based on the same cohort of patients as in the C-PEPS study.

Patients' median age was 80 years. Atrial fibrillation occurred in 26 stroke / TIA patients (27%) and 17 control medical patients (24%). Ischaemic ECG changes occurred in 54 stroke / TIA patients (56%) and 32 control medical patients (46%) (OR= 1.52; 95% CI= 0.82 to 2.83; P= 0.18). 17 stroke / TIA patients (18%) versus 19

control medical patients (27%) had history of IHD. After adjustment for IHD, the OR in relation to stroke / TIA for ischaemic ECG changes was 1.80 (95% CI= 0.93 to 3.45; P= 0.079).

This study found that atrial fibrillation accounted for a quarter of ECG rhythms in elderly, acute stroke / TIA and medical patients. The rate of atrial fibrillation in this study was amongst the highest in any published series. The high frequency of ischaemic ECG changes found in the stroke / TIA patients was not significantly different from that in the control medical patients. However, after adjustment for IHD, there emerged a statistical trend of borderline significance to suggest that ischaemic ECG changes were more strongly associated with elderly, acute stroke / TIA patients than control medical patients. The prognostic significance of various ECG abnormalities, sequential monitoring and possible intervention in the context of acute cerebrovascular events in elderly patients would merit further studies.

#### **9.11. Socioeconomic deprivation, atypical respiratory infections, survival outcome and stroke**

Previous studies have suggested a relationship between socioeconomic deprivation (SED), stroke and infections. An opportunistic study was conducted to investigate the relationship between SED, atypical respiratory infections and survival outcome in elderly stroke and medical patients that came from the same cohort of patients as in the C-PEPS study. The socioeconomic status of 92 ischaemic stroke / TIA patients and 87 control medical patients were determined using the English Index of Multiple Deprivation (IMD) and Income Deprivation Affecting Older People Index (IDAOPI)

deprivation scores which were updated and published by the UK government in 2004. Patients' survival was determined from their health records after 6-years of follow-up.

The mean IMD scores of stroke / TIA patients and control medical patients were 23.3 and 25.0, respectively (difference= 1.7;  $P= 0.29$ ). The mean IDAOPI scores of stroke / TIA patients and control medical patients were 0.19 and 0.21, respectively (difference= 0.02;  $P= 0.22$ ). The socioeconomic status of elderly stroke / TIA and medical patients was therefore similar i.e. no significant differences in their mean SED scores.

In the control medical patients, but not the stroke / TIA patients, there was a statistical difference in the mean IMD ( $P= 0.02$ ) and IDAOPI ( $P= 0.005$ ) scores between patients seropositive and seronegative for IgM, but not for IgG. This suggested that acute atypical respiratory infection could be associated with SED in the control medical patients. There was also an association between number of pathogens (IgM but not IgG) and IDAOPI scores ( $P= 0.004$ ), suggesting that acute atypical respiratory infectious burden was significantly and specifically associated with income deprivation in both the elderly stroke / TIA and control medical patients.

The association between stroke / TIA and IgG seropositivity for atypical respiratory infectious burden was significant before adjustment ( $P= 0.04$ ) and close to significance after adjusting for IMD ( $P= 0.052$ ) and IDAOPI ( $P= 0.054$ ). SED therefore has only a modest effect on the association between chronic atypical respiratory infectious burden and elderly stroke / TIA patients.

After 6-years of follow-up, 69 out of the 92 stroke / TIA patients (75%) (median survival= 22.4 months) and 68 out of the 87 control medical patients (78%) (median survival= 36.5 months) died. There was no significant difference between the Kaplan-Meier survival curves for stroke / TIA patients and control medical patients, and as divided into tertiles of IMD and IDAOPI deprivation scores. Therefore, SED was not found to affect elderly patients' survival in this study.

## 9.12. Conclusions

Acute infections due to specific micro-organisms causing stroke events are well recognised. Chronic infections caused by specific micro-organisms are also known to associate with stroke. It has been hypothesised that specific chronic infections could contribute to atherosclerosis through chronic inflammatory and immunological mechanisms. *Chlamydia pneumoniae*, an atypical respiratory pathogen, is the micro-organism most investigated in this model of infectious hypothesis of atherosclerosis. Although the evidence as a whole is compelling, definitive proof of a causal relationship between *C pneumoniae* and atherosclerosis or stroke remains elusive.

In accordance with the infectious hypothesis of atherosclerosis, it is likely that chronic infections by a number of specific micro-organisms would contribute to atherogenesis and atherothrombosis. This thesis has established the seroprevalences of individual atypical respiratory pathogens i.e. *C pneumoniae*, *M pneumoniae*, *L pneumophila* and *C burnetii*, in a cohort of hospitalised, elderly acute stroke / TIA and control medical patients. This thesis has shown that rather than any individual micro-organism, an aggregate number (infectious burden) of specific chronic atypical respiratory

infections i.e. *C pneumoniae*, *M pneumoniae*, *L pneumophila* was more likely to associate with stroke / TIA. Anti-microbial intervention, ideally targeted to specific infectious burden in susceptible individuals, and immunisation studies, in the context of chronic atypical respiratory infections and stroke prevention, are possible areas for future research studies.

This thesis has established the reproducibility of SeroCP ELISA kits used in the C-PEPS study. The reproducibility was good in the detection of *C Pneumoniae* IgA, and it was moderately good in the detection of *C pneumoniae* IgG and IgM. The reproducibility study has highlighted an important issue of laboratory test limitation or reliability in seroepidemiological studies. Further standardisation, refinement and improvement in serological and laboratory techniques have been proposed.

It has been documented that as frequently as one in four elderly patients has ECG evidence of atrial fibrillation on their acute hospital admission. This thesis has established that there was a statistical trend to suggest that ischaemic ECG changes in elderly stroke / TIA patients could be due to the underlying acute cerebrovascular events, independent of their background history of ischaemic heart disease. Future outcome study may be required to establish the significance of ischaemic ECG changes and other ECG abnormalities in the context of acute cerebrovascular events in older patients.

Finally, this thesis has established that the socioeconomic status of elderly stroke / TIA and general medical patients was similar on their acute hospital admission; and that their 6-year survival outcome was similar. It has further established that acute



atypical respiratory infectious burden was associated with income deprivation in hospitalised elderly stroke / TIA and medical patients. Future study may be required to test the hypothesis that if measures to improve elderly patients' socioeconomic status or income would reduce the rate of acute atypical respiratory infections in these patients. Although this thesis established that socioeconomic status has only a modest effect on the association between chronic atypical respiratory infections and stroke / TIA, it has recommended that socioeconomic deprivation (SED), particularly income deprivation, should be adjusted for in similar future studies. As SED, infections and stroke are potentially important modifiable risk factors in older patients, adequately powered interventional and outcome studies to explore the relationship between these factors are potential areas for future research.

## Appendix A

### Principle and procedure of Enzyme-Linked Immunosorbent Assay (ELISA) test

1. Microtitre plates or microwells are supplied coated with purified antigens i.e. *C pneumoniae*<sup>\*</sup> TW<sup>TM</sup>-183 elementary bodies, *M pneumoniae*<sup>†</sup> P1-enriched membrane antigens, *L pneumophila*<sup>‡</sup> serogroup-1 LPS antigens or *C burnetii*<sup>§</sup> Phase-II antigens.
2. The serum to be tested is diluted (e.g. 1/21<sup>\*</sup>, 1/100<sup>§</sup> or 1/105<sup>\*†</sup>). For IgM testing, the serum is pre-treated with the manufacturer's serum diluent containing anti-IgG absorbent, to remove rheumatoid factor (i.e. to reduce false positive result), and to reduce excessive IgG interference (i.e. to reduce false negative result).
3. 50<sup>\*†</sup>, 100<sup>§</sup>, or 105<sup>‡</sup> µL diluted serum is incubated in the microtitre plate for 30<sup>§</sup>, 45<sup>‡</sup> or 60<sup>\*†</sup> minutes at 37±1°C. In this step, specific antibodies in the serum tested are bound to the immobilised antigens attached to the polystyrene surface of the microwells.
4. Non-specific or unbounded antibodies in the residual serum are removed by washing with Wash Buffer 3<sup>\*†</sup>, 5<sup>‡</sup> or 6<sup>§</sup> times.
5. Anti-human IgA, IgG or IgM conjugated to Horseradish Peroxidase (HRP) is diluted e.g. 1/300<sup>\*†</sup> with a conjugate diluent. Dispense 50<sup>\*†</sup> or 100<sup>‡§</sup> µL of diluted conjugate solution into each well. Cover and incubate for 30<sup>‡§</sup> or 60<sup>\*†</sup> minutes at 37±1°C in a moisture chamber. In this step, the HRP-Ig-Conjugate is bound to the prebound antigen-antibody complex formed in step 2 above.

6. Unbound conjugate is removed by washing with Wash Buffer 3<sup>\*†</sup>, 5<sup>‡</sup> or 6<sup>§</sup> times.
7. Upon the addition of 100 µL colourless Tetramethylbenzidine<sup>\*†‡</sup>/Hydrogen Peroxide-Substrate<sup>§</sup> (TMB/H<sub>2</sub>O<sub>2</sub>-Substrate), the Substrate is hydrolysed by the peroxidase (HRP), yielding a blue solution of the reduced Substrate. Cover plates and incubate at room temperature (20-25°C) for 10<sup>§</sup>, 15<sup>\*†</sup> or 20<sup>‡</sup> minutes.
8. Upon the addition of 50<sup>‡</sup> or 100<sup>\*†§</sup> µL acid Stop Solution (contains 0.5<sup>‡</sup> or 1<sup>\*†§</sup> M sulphuric<sup>\*†‡</sup> or phosphoric<sup>§</sup> acid), the blue colour turns yellow and should be read by an ELISA reader at a wavelength of 450nm with a reference filter of 620nm, within 30<sup>\*†§</sup> or 60<sup>‡</sup> minutes of stopping.
9. The absorbance or optical density (OD) read is proportional to the amount of the specific antibodies that are bound to the coated antigens.
10. Calculate and interpret results. See Appendix B.

\* refers to *Chlamydia pneumoniae*-SeroCP ELISA (Savyon, Israel)

† refers to *Mycoplasma pneumoniae*-SeroMP ELISA (Savyon, Israel)

‡ refers to *Legionella pneumophila*-ELISA (VIRCELL SL, Spain)

§ refers to *Coxiella burnetii*-ELISA (PANBIO, Australia)

## Appendix B

### ELISA test validation, calculation and interpretation of results

#### *Chlamydia pneumoniae*-SeroCP ELISA (Savyon, Israel)

1. For the test to be valid, two criteria must be met:
  - a. the absorbance value or optical density **OD<sub>450</sub>** of the positive control should be  $\geq 0.8$  at 450nm,
  - b. the **average OD<sub>450</sub>** of the two negative controls **NC** should be  $0.1 < NC \leq 0.4$ .

2. The Cut-Off Value (COV) is calculated using the formula:

$$\text{COV} = (\text{the average OD}_{450} \text{ of the negative control run in duplicate}) \times 2$$

3. In order to normalise the results obtained in different tests, the Cut-Off Index (COI) is calculated according to the formula:

$$\text{COI} = \text{OD}_{450} \div \text{COV}$$

4. As defined by the manufacturer:

**COI < 1.0 = negative** i.e. no detectable antibodies,

**COI = 1-1.1 = borderline** i.e. low level of antibodies, second sample testing recommended,

**COI > 1.1 = positive** i.e. relevant level of antibodies.

### ***Mycoplasma pneumoniae*-SeroMP ELISA (Savyon, Israel)**

1. The following criteria must be met for the test to be valid:

a. **OD<sub>P100</sub> > 1.2**

The absorbance value or optical density OD at 450/620nm for the Calibrator P100 containing 100 Ig BU/ml (immunoglobulin binding units per ml of serum) should be greater than 1.2.

b. Ratio: **OD<sub>P10</sub> / OD<sub>NC</sub> > 1.5**

The ratio of the OD<sub>450/620</sub> for the Calibrator P10 containing 10 Ig BU/ml and the OD<sub>450/620</sub> for the negative control NC should be greater than 1.5.

c. Ratio: **OD<sub>P50</sub> / OD<sub>NC</sub> > 4**

OD<sub>P50</sub> is the OD<sub>450/620</sub> for the Calibrator P50 containing 50 Ig BU/ml. OD<sub>NC</sub> is the OD<sub>450/620</sub> for the negative control.

d. Ratio: **OD<sub>P100</sub> / OD<sub>NC</sub> > 7**

2. Using a squared graph paper, plot the OD<sub>450/620</sub> of the 3 calibrators P10, P50 and P100 on Y-axis (OD<sub>450/620</sub>) versus their concentration i.e. 10, 50, 100 BU/ml, respectively, on X-axis.

3. Draw the best fitted linear curve through the points.

4. Using the standard curve, interpolate the concentration of the tested sample values in BU/ml (on X-axis) from each absorbance or OD (on Y-axis) measured.

5. As defined by the manufacturer, concentration value of:

**< 10 BU/ml = negative** i.e. no detectable antibodies,

**≥ 10 BU/ml = positive** i.e. relevant level of antibodies,

**≥ 50 BU/ml = high positive** i.e. high level of antibodies.

***Legionella pneumophila*-ELISA (VIRCELL SL, Spain)**

1. For the test to be valid, the following criteria must be met:
  - a. the optical density **OD<sub>450/620</sub> of the positive control should be > 0.9**,
  - b. the **OD<sub>450/620</sub> of the negative control should be < 0.55**,
  - c. the **OD<sub>450/620</sub> of the cut-off control should be < 0.7 X (OD positive control) and > 1.5 X (OD negative control)**.
2. Calculate the mean OD for the 2 cut-off serum controls.
3. **Antibody Index = (sample OD ÷ cut-off serum mean OD) X 10**
4. As defined by the manufacturer, **Antibody Index** of:  
**< 9 = negative** i.e. no detectable antibodies,  
**9-11 = equivocal**, retesting/new sample obtained for confirmation recommended,  
**> 11 = positive** i.e. antibodies detected

***Coxiella burnetii*-ELISA (PANBIO, Australia)**

1. Each kit contains cut-off calibrator, positive and negative control sera.
2. Acceptable values for these sera are found on the accompanying specification sheet.
3. The negative and positive controls are intended to monitor for substantial reagent failure.
4. The test is invalid and should be repeated if the absorbance readings of either the controls or the calibrator do not meet the specifications.
5. The cut-off value COV is the average absorbance of the triplicates of the cut-off calibrator.
6. A sample's **Index Value** = **Sample Absorbance** ÷ **COV**
7. **PANBIO Units** = **Index Value X 10**
8. As defined by the manufacturer, **PANBIO Units** of:  
< **9** = **negative** i.e. no detectable antibodies,  
**9-11** = **equivocal**, retesting/new sample obtained for confirmation recommended,  
> **11** = **positive** i.e. antibodies detected.

## Appendix C

### List of relevant oral and poster presentations, and peer-reviewed abstract publications

1. Prevalence of electrocardiographic (ECG) abnormalities in acute stroke and medical patients- **poster** presentation at the British Geriatrics Society Spring Meeting, 10-12 April 2003, in Aberdeen.

1.1. Ngeh J. Prevalence of electrocardiographic (ECG) abnormalities in acute stroke and medical patients. **Age and Ageing 2003;32-S2:19.**

2. Seroprevalence of *Mycoplasma pneumoniae* in elderly stroke and medical patients: a nested case-control study- **oral**, platform presentation at the British Geriatrics Society Autumn Meeting, 18-19 October 2001, in London.

2.1. Ngeh J, Gupta S, Goodbourn C, McElligott G. Seroprevalence of *Mycoplasma pneumoniae* in elderly stroke and medical patients: a nested case-control study. **Age and Ageing 2002;31-S1:45.**

3. Seroprevalence of *Chlamydia pneumoniae* in elderly stroke and medical patients: a case-control study- **oral**, platform presentation at the Regional British Geriatrics Society Meeting (Joint North-East and North-West Thames), 4 July 2001, RAF Museum, Graham Park Way, Colindale, London NW9 5LL.

4. Seroprevalence of *Chlamydia pneumoniae* in elderly stroke and medical patients: a case-control study- **poster** presentation at the British Geriatrics Society Spring Meeting, 5-7 April 2001, in Cardiff. Awarded **Ferguson Anderson Best Poster Prize, 2001.**

4.1. Ngeh J, Gupta S, Goodbourn C, Panayiotou B, McElligott G. Seroprevalence of *Chlamydia pneumoniae* in elderly stroke and medical patients: a case-control study. **Age and Ageing 2001;30-S2:60.**



## Bibliography

Ahto M, Isoaho R, Puolijoki H, Laippala P, Romo M, Kivela SL 1998. Prevalence of coronary heart disease, associated manifestations and electrocardiographic findings in elderly Finns. *Age Ageing* 27:729-737.

Aimetti M, Romano F, Nessi F 2007. Microbiological analysis of periodontal pockets and carotid atheromatous plaques in advanced chronic periodontitis patients. *J Periodontol* 78:1718-1723.

Airenne S, Surcel HM, Tuukkanen J, Leinonen M, Saikku P 2002. *Chlamydia pneumoniae* inhibits apoptosis in human epithelial and monocyte cell lines. *Scand J Immunol* 55:390-398.

Alamowitch S, Labreuche J, Touboul PJ, Eb F, Amarenco P 2008. *Chlamydia pneumoniae* seropositivity in etiologic subtypes of brain infarction and carotid atherosclerosis: a case-control study. *J Neurol Neurosurg Psychiatry* (doi:10.1136/jnnp.2007.126862) 79:147-151.

Alcock P 2007. Updating the English indices of deprivation 2004: stage two 'blueprint' peer review. Department for Communities and Local Government Publications, London, UK.

Available at: <http://www.communities.gov.uk/documents/communities/pdf/321700>

Al-Shahi Rustam 2000. Stroke. *Journal of the Royal College of Physicians of London* 34(1): 97-99.

Ameriso SF, Fridman EA, Leiguarda RC, Sevlever GE 2001. Detection of *Helicobacter pylori* in human carotid atherosclerotic plaques. *Stroke* 32:385-391.

Ameriso SF, Wong VL, Quismorio FP, Fisher M 1991. Immunohaematologic characteristics of infection-associated cerebral infarction. *Stroke* 22(8): 1004-1009.

Anderson JL 2005. Infection, antibiotics, and atherothrombosis- end of the road or new beginnings? N Engl J Med 352:1706-1709.

Anderson JL, Muhlestein JB, Carlquist J et al 1999. Randomised secondary prevention trial of azithromycin in patients with coronary artery disease and serological evidence for *Chlamydia pneumoniae* infection. The Azithromycin in Coronary Artery Disease: Elimination of Myocardial Infection with Chlamydia (ACADEMIC) Study. Circulation: 99:1540-1547.

Andr ws R, Berger JS, Brown DL 2005. Effects of antibiotic therapy on outcomes of patients with coronary artery disease: a meta-analysis of randomized controlled trials. JAMA 293:2641-2647.

Anonymous 1989. Uncommon causes of stroke. Lancet 1:26.

Antachopoulos C, Liakopoulou T, Palamidou F, Papathanassiou D, Youroukos S 2002. Posterior cerebral artery occlusion associated with *Mycoplasma pneumoniae* infection. J Child Neurol 17:55-57.

Anzini A, Cassone A, Rasura M, Ciervo A, Beccia M, Di Lisi F, Fieschi C 2004. *Chlamydia pneumoniae* infection in young stroke patients: a case-control study. Eur J Neurol 11:321-327.

Apfalter P 2006. *Chlamydia pneumoniae*, stroke, and serological associations: anything learned from the atherosclerosis-cardiovascular literature or do we have to start over again? Stroke 37:756-758.

Arrich J, Lalouschek W, Mullner M 2005. Influence of socioeconomic status on mortality after stroke- retrospective cohort study. Stroke 36:310-314.

Arthur G, Margolis G 1977. *Mycoplasma*-like structures in granulomatous angiitis of the central nervous system. Arch Pathol 101:385-387.

Aslanyan S, Weir CJ, Lees KR, Reid JL, McInnes GT 2003. Effect of area-based deprivation on the severity, subtype, and outcome of ischaemic stroke. *Stroke* 34:2523-2629.

Auer JW, Berent R, Weber T, Eber B 2002. Immunopathogenesis of atherosclerosis. *Circulation* 105:e64.

Ay H, Koroshetz WJ, Benner T, Vangel MG, Melinosky C, Arsava EM, Ayata C, Zhu M, Schwamm LH, Sorensen AG 2006. Neuroanatomic correlates of stroke-related myocardial injury. *Neurology* 66:1325-1329.

Badimon L, Chesebro JH, Badimon JJ 1992. Thrombus formation on ruptured atherosclerotic plaques and rethrombosis on evolving thrombi. *Circulation* 86 (suppl III):74-85.

Bamford J, Sandercock P, Dennis M, Warlow C 1990. A prospective study of acute cerebrovascular disease in the community: the Oxfordshire Community Stroke Project II. Incidence, case fatality rates and overall outcome at one year of cerebral infarction, primary intracerebral and subarachnoid haemorrhage. *J Neurol Neurosurg Psychiatry* 53: 16-22.

Barber M, Morton JJ, Macfarlane PW, Barlow N, Roditi G, Stott DJ 2007. Elevated troponin levels are associated with sympathoadrenal activation in acute ischaemic stroke. *Cerebrovasc Dis* 23:260-266.

Barka N, Tomasi J-P, Stadtsbaeder S 1986. ELISA using whole *Legionella pneumophila* cell as antigen: comparison between monovalent and polyvalent antigens for the serodiagnosis of human legionellosis. *Journal of Immunological Methods* 93:77-81.

Barone FC, Arvin B, White RF et al 1997. Tumour necrosis factor- $\alpha$ : a mediator of focal ischaemic brain injury. *Stroke* 28:1233-1244.

Bartolome J, Riquelme E, Hernandez-Perez N, Garcia-Ruiz S, Lujan R, Lorente S, Medrano-Callejas R, Crespo MD 2007. Seroepidemiology of *Coxiella burnetii* infection among blood donors in Albacete. *Enferm Infecc Microbiol Clin* 25:382-386.

Basinkevich AB, Shakhnovich RM, Martynova VR, Kolkova NI, Rakovskaya IV, Karazhas NV, Evseeva LF, Dobrovolsky AB, Deev AD, Noeva EA, Ruda MI 2003. Role of Chlamydia, Mycoplasma and Cytomegalovirus infection in the development of coronary artery disease. *Kardiologiia* 43:4-9.

Beck J, Garcia R, Heiss G, Vokonas PS, Offenbacher S 1996. Periodontal disease and cardiovascular disease. *J Periodontol* 67:1123-1137.

Beersma MF, Dirven K, van Dam AP, Templeton KE, Class EC, Goossens H 2005. Evaluation of 12 commercial tests and the complement fixation test for *Mycoplasma pneumoniae*-specific immunoglobulin G (IgG) and IgM antibodies, with PCR used as the 'gold standard'. *J Clin Microbiol* 43:2277-2285.

Benito-Leon J, Guerrero AL, Simon R, Mateos F 1998. Ischaemic stroke in children (in Spanish). *Revista de Neurologia* 27:631-634.

Ben-Shlomo Y, Davey-Smith G 1991. Deprivation in infancy and in adult life: which is more important for mortality risk? *Lancet* 337:530-534.

Berenson GS, Radhakrishnamurthy B, Srinivasan SR et al 1984. Carbohydrate-protein macromolecules and arterial wall integrity – a role in atherogenesis. *Exp Mol Pathol* 41:267-287.

Berg HF, Maraha B, Scheffer G-J, Quarles-van Ufford M, Vandenbroucke-Grauls CM, Peeters MF et al 2005(a). Treatment with clarithromycin prior to coronary artery bypass graft surgery does not prevent subsequent cardiac events. *Clin Infect Dis* 40:358-365.

Berg HF, Maraha B, van der Zee A, Gielis SK, Roholl PJM, Scheffer GJ, Peeters MF, Kluytmans JAJW 2005(b). Effect of clarithromycin treatment on *Chlamydia pneumoniae* in vascular tissue of patients with coronary artery disease: a randomized, double-blind, place-controlled trial. *Journal of Clinical Microbiology* 43:1325-1329.

Bernit E, Pouget J, Janbon F, Dutronc H, Martinez P, Brouqui P, Raoult D 2002. Neurological involvement in acute Q fever- a report of 29 cases and review of the literature. *Arch Intern Med* 162:693-700.

Bickerstaff ER 1964. Aetiology of acute hemiplegia in childhood. *BMJ* 2: 82-87.

Blessing E, Campbell LA, Rosenfeld ME, Chough N, Kuo C-C 2001. *Chlamydia pneumoniae* infection accelerates hyperlipidemia induced atherosclerotic lesion development in C57BL/6J mice. *Atherosclerosis* 158:13-17.

Bloemenkamp DGM, Mali WPTM, Tanis BC, Rosendaal FR, van den Bosch AAJ, Kemmeren JM, Algra A, Ossewaarde JM, Visseren FLJ, van Loon AM, van der Graaf Y 2002. *Chlamydia pneumoniae*, *Helicobacter pylori* and cytomegalovirus infections and the risk of peripheral arterial disease in young women. *Atherosclerosis* 163:149-156.

Boman J, Hammerschlag MR 2002. *Chlamydia pneumoniae* and atherosclerosis: critical assessment of diagnostic methods and relevance to treatment studies. *Clinical Microbiology Reviews* 15: 1-20.

Bondjiers G, Wiklund O, Fager G et al 1990. Transfer of lipoprotein from plasma to the cell population of the normal and atherosclerotic arterial tissue. *Eur Heart J Suppl* E:158-163.

Bone RC 1992. Modulators of coagulation: a critical appraisal of their role in sepsis. *Arch Intern Med* 152: 1381-1389.

Bornstein NM, Bova IY, Korczyn AD 1997. Infections as triggering factors for ischaemic stroke. *Neurology* 49(Suppl 4): S45-S46.

Bourdette DN, Rosenberg NL, Yatsu FM 1983. Herpes zoster ophthalmicus and delayed ipsilateral cerebral infarction. *Neurology* 33: 1428-1432.

Bova IY, Bornstein NM, Korczyn AD 1996. Acute infection as a risk factor for ischaemic stroke. *Stroke* 27:2204-2206.

Bowling A 2004. Socioeconomic differentials in mortality among older people. *J Epidemiol Community Health* 58:438-440.

Bozluolcay M, Ince B, Celik Y, Harmanci H, Ilerigelen B, Pelin Z 2003. Electrocardiographic findings and prognosis in ischaemic stroke. *Neurol India* 51:500-502.

Brassard P, Bourgault C, Brophy J, Kezouh A, Rainville B, Xhignesse M, Suissa S 2003. Antibiotics in primary prevention of myocardial infarction among elderly patients with hypertension. *Am Heart J* 145:E20.

Brassard P, Bourgault C, Brophy J, Kezouh A, Suissa S 2003. Antibiotics in primary prevention of stroke in the elderly. *Stroke* 34:e163-e166.

Breeze E, Jones DA, Wilkinson P, Bulpitt CJ, Grundy C, Latif AM, Fletcher AE 2005. Area deprivation, social class, and quality of life among people aged 75 years and over in Britain. *Int J Epidemiol* 34:276-283.

Bruggeman CA 1993. Cytomegalovirus and latency: an overview. *Virchows. Arch B Cell Pathol Incl Mol Pathol* 64:325-333.

Bucurescu G, Stieritz DD 2003. Evidence of an association between *Chlamydia pneumoniae* and cerebrovascular accidents. *European Journal of Neurology* 10:449-452.

Campbell LA, Kuo CC 1999. Mouse models of *Chlamydia pneumoniae* infection. *Am Heart J* 138:S516-518.

Campbell LA, Moazed TC, Kuo CC, Grayston JT 1998. Preclinical models for *Chlamydia pneumoniae* and cardiovascular disease: hypercholesterolaemic mice. Clin Microbiol Infect Suppl 4:S23-S32.

Cannon CP, Braunwald E, McCabe CH, Grayston JT, Muhlestein B, Giugliano RP, Cairns R, Skene AM, for the Pravastatin or Atorvastatin Evaluation and Infection Therapy-Thrombolysis in Myocardial Infarction 22 Investigators 2005. Antibiotic treatment of *Chlamydia pneumoniae* after acute coronary syndrome. N Engl J Med 352:1646-1654.

Cardenosa N, Sanfeliu I, Font B, Munoz T, Nogueras MM, Segura F 2006. Short report: seroprevalence of human infection by *Coxiella burnetii* in Barcelona (northeast of Spain) Am J Trop Med Hyg 75:33-35.

Carlos TM, Harlan JM. Leucocyte-endothelial adhesion molecules 1994. Blood 84: 2068-2101.

Carstairs V, Morris R 1991. Deprivation and health in Scotland. Aberdeen, Scotland: Aberdeen University Press.

Carter AM, Catto AJ, Mansfield MW, Bamford JM, Grant PJ 2007. Predictive variables for mortality after acute ischemic stroke. Stroke 38:1873-1880.

Cercek B, Shah PK, Noc M, Zahger D, Zeymer U, Matetzky S, Maurer G, Mahrer P 2003. Effect of short-term treatment with azithromycin on recurrent ischaemic events in patients with acute coronary syndrome in the Azithromycin in Acute Coronary Syndrome (AZACS) trial: a randomised controlled trial. Lancet 361:809-13.

Chiu B 1999. Multiple infections in carotid atherosclerotic plaques. American Heart Journal 138(5 Pt 2): S534-536.

Christensen H, Fogh Christensen A, Boysen G 2005. Abnormalities on ECG and telemetry predict stroke outcome at 3 months. *J Neurol Sci* 234:99-103.

Chunn CJ, Jones SR, McCutchan JA et al 1977. *Haemophilus parainfluenzae* infective endocarditis. *Medicine* 56: 115-128.

Ciervo A, Petrucca A, Visca P, Cassone A 2004. Evaluation and optimization of ELISA for detection of anti-*Chlamydia pneumoniae* IgG and IgA in patients with coronary heart diseases.

Clayton TC, Thompson M, Meade TW 2008. Recent respiratory infection and risk of cardiovascular disease: case-control study through a general practice database. *European Heart Journal* 29:96-103.

Clowes AW, Clowes MM, Fingerle J et al 1989. Regulation of smooth muscle cell growth in injured artery. *J Cardiovasc Pharmacol* 14(suppl 6):12-15.

Coles KA, Plant AJ, Riley TV, Smith DW, McQuillan BM, Thompson PL 1999. Lack of association between seropositivity to *Chlamydia pneumoniae* and carotid atherosclerosis. *Am J Cardiol* 84:825-828.

Cook PJ, Honeybourne D, Lip GYH, Beevers DG, Wise R, Davies P 1998. *Chlamydia pneumoniae* antibody titres are significantly associated with acute stroke and transient cerebral ischaemia. The West Birmingham Stroke Project. *Stroke* 29:404-410.

Corrado E, Novo S 2005. Role of inflammation and infection in vascular disease. *Acta Chir Belg* 105:567-579.

Corrado E, Rizzo M, Tantillo R, Muratori I, Bonura F, Vitale G, Novo S 2006. Markers of inflammation and infection influence the outcome of patients with baseline asymptomatic carotid lesions: a 5-year follow-up study. *Stroke* 37:482-486.



Cotter ME, Krakover R, Cotter G, Ben YM, Lazarovich T, Chen LZ, Zaidenstein R, Fytlovich S, Boldur I, Golik A 2003. Cumulative infectious burden (but not any single past infection) is interrelated with traditional risk factors to cause coronary artery disease. J Am Coll Cardiol 41(6 Suppl B),P:344.

Cowley R, Fernandez F, Freemantle W, Rutter D 1992. Enzyme immunoassay for Q fever: comparison with complement fixation and immunofluorescence tests and dot immunoblotting. J Clin Microbiol 30:2451-2455.

Cox AM, McKeivitt C, Rudd AG, Wolfe CDA 2006. Socioeconomic status and stroke. Lancet Neurol 5:181-188.

Cremonini F, Gabrielli M, Gasbarrini G, Pola P, Gasbarrini A 2004. The relationship between chronic *H pylori* infection, *CagA* seropositivity and stroke: meta-analysis. Atherosclerosis 173:253-259.

Dahlen GH, Boman J, Birgander LS, Lindblom B 1995. Lp(a) lipoprotein, IgG, IgA and IgM antibodies to *Chlamydia pneumoniae* and HLA class II genotype in early coronary artery disease. Atherosclerosis 114:165-174.

D'Aiuto F, Parkar M, Andreou G, Brett PM, Ready D, Tonetti MS 2004(a). Periodontitis and atherogenesis: causal association or simple coincidence? J Clin Periodontol 31:402-411.

D'Aiuto F, Parkar M, Andreou G et al 2004(b). Periodontitis and systemic inflammation: control of the local infection is associated with a reduction in serum inflammatory markers. J Dent Res 83:156-160.

D'Aiuto F, Nibali L, Parkar M, Suvan J, Tonetti MS 2005. Short-term effects of intensive periodontal therapy on serum inflammatory markers and cholesterol. J Dent Res 84:269-273.

D'Aiuto F, Parkar M, Nibali L, Suvan J, Lessem J, Tonetti MS 2006. Periodontal infections cause changes in traditional and novel cardiovascular risk factors: results from a randomized controlled clinical trial. *Am Heart J* 151:977-984.

Dalal PM, Dalal KP 1989. Cerebrovascular manifestations of infectious disease. In: Vinken PJ, Bruyn GW, Klawans HL, eds. *Handbook of Clinical Neurology, Vol. II: Vascular Diseases, Part III*. Amsterdam: Elsevier, 411-441.

Danesh J, Collins R, Peto R 1997. Chronic infections and coronary heart disease: is there a link? *Lancet* 350:430-436.

Davey-Smith G, Hart C, Blane D, et al 1998. Adverse socioeconomic conditions in childhood and cause specific adult mortality: prospective observational study. *BMJ* 316:1631-1635.

Davey-Smith G, Whitley E, Dorling D, Gunnell D 2001. Area based measures of social and economic circumstances: cause specific mortality patterns depend on the choice of index. *J Epidemiol Community Health* 55:149-150.

Davies MJ, Woolf N 1993. Atherosclerosis: what is it and why does it occur? *Br Heart J* 69 (suppl):3-11.

Davies PF, Dewey CF, Bussolari SR et al 1984. Influence of haemodynamic forces on vascular endothelial function. In vitro studies of shear stress and pinocytosis in bovine aortic cells. *J Clin Invest* 73:1121-1129.

Davis TP, Alexander J, Lesch M 1993. Electrocardiographic changes associated with acute cerebrovascular disease: a clinical review. *Progress in Cardiovascular Diseases* 36:245-260.

Davies TR, Edwards Y, Morgan A, Caul EO 1997. Prevalence of Q fever in a rural practice. *J Public Health Med* 19:324-327.

Daxboeck F, Kircher K, Krause R, Heinzl H, Wenisch C, Stanek G 2002. Effect of age on antibody titer to *Mycoplasma pneumoniae*. Scand J Infect Dis 34:577-579.

De Bacquer D, De Backer G 2002. Electrocardiographic findings and global coronary risk assessment. European Heart Journal 23:268-270.

De Bacquer D, De Backer G, Kornitzer M 2000. Prevalences of ECG findings in large population based samples of men and women. Heart 2000;84:625-633.

Dechend R, Maass M, Gieffers J 1999. *Chlamydia pneumoniae* infection of vascular smooth muscle and endothelial cells activates NF-kappaB and induces TF and PAI-1 expression: a potential link to accelerated arteriosclerosis. Circulation 100:1369-1373.

Del Brutto OH 1992. Cysticercosis and cerebrovascular disease: a review. J Neurol Neurosurg Psychiatr 55: 252-254.

Desvarieux M, Schwahn C, Volzke H et al 2004. Gender differences in the relationship between periodontal disease, tooth loss, and atherosclerosis. Stroke 35:2029-2035.

Diederens BMW, Kluytmans JAJW, Peeters MF 2006. Evaluation of Vircell enzyme-linked immunosorbent assay and indirect immunofluorescence assay for detection of antibodies against *Legionella pneumophila*. Clinical and Vaccine Immunology 13:361-364.

Diez Roux AV, Borrell LN, Haan M, Jackson SA, Schultz R 2004. Neighbourhood environments and mortality in an elderly cohort: results from the cardiovascular health study. J Epidemiol Community Health 58:917-923.

Dimant J, Grob D 1977. Electrocardiographic changes and myocardial damage in patients with acute cerebrovascular accidents. Stroke 8:448-455.

Dowd AB, Grace R, Rees WDW 1987. Cerebral infarction associated with *Mycoplasma pneumoniae* infection. Lancet 2:567.

Dowell SF, Peeling RW, Boman J, Carlone GM, Fields BS, Guarner J, Hammerschlag MR, Jackson LA, Kuo CC, Maass M, Messmer TO, Talkington DF, Tondella ML, Zaki SR, *C pneumoniae* Workshop Participants 2001. Standardizing *Chlamydia pneumoniae* assays: recommendations from the Centers for Disease Control and Prevention (USA) and the Laboratory Centre for Disease Control (Canada). *Clinical Infectious Diseases* 33:492-503.

Dugan JP, Feuge RR, Burgess DS 2002. Review of evidence for a connection between *Chlamydia pneumoniae* and atherosclerotic disease. *Clinical Therapeutics* 24:719-735.

Duguid JB 1946. Thrombus as a factor in the pathogenesis of coronary atherosclerosis. *J Pathol Bacteriol* 58:207

Duncan GJ, Daly MC, McDonough P, Williams DR 2002. Optimal indicators of socioeconomic status for health research. *Am J Public Health* 92:1151-1157.

Easterbrook PJ, Smyth EG 1992. Post-infectious encephalomyelitis associated with *Mycoplasma pneumoniae* and *Legionella pneumophila* infection. *Postgrad Med J* 1992;68:124-128.

Ebrahim S 1998. Stroke: pathology and epidemiology. In: Tallis RC, Fillit HM, Brocklehurst JC (5<sup>th</sup> edition). *Textbook of Geriatric Medicine and Gerontology*. Churchill Livingstone, 487-498.

Einarsson S, Sigurdsson HK, Magnúsdóttir SD et al 1994. Age specific prevalence of antibodies against *Chlamydia pneumoniae* in Iceland. *Scand J Infect Dis* 26:393-397.

Elkind MSV, Lin I-F, Grayston JT, Sacco RL 2000. *Chlamydia pneumoniae* and the risk of first ischaemic stroke: The Northern Manhattan Stroke Study. *Stroke* 31:1521-1525.

Elkind MSV, Cole JW 2006. Do common infections cause stroke? *Seminars in Neurology* 26:88-99.

Elkind MSV, Tondella MLC, Feikin DR, Fields BS, Homma S, Di Tullio MR 2006. Seropositivity to *Chlamydia pneumoniae* is associated with risk of first ischemic stroke. *Stroke* 37:790-795.

Elkins JS, Sidney S, Gress DR, Go AS, Bernstein AL, Claiborne Johnston S 2002. Electrocardiographic findings predict short-term cardiac morbidity after transient ischemic attack. *Arch Neurol* 59:1437-1441.

Elneihoum AM, Falke P, Axelsson L et al 1996. Leucocyte activation detected by increased plasma levels of inflammatory mediators in patients with ischaemic cerebrovascular diseases. *Stroke* 27: 1734-1738.

Engelbreton SP, Lamster IB, Elkind MSV et al 2005. Radiographic measures of periodontal disease and carotid atherosclerosis. *Stroke* 36:561-566.

Esmon CT, Taylor FB, Snow TR 1991. Inflammation and coagulation: linked processes potentially regulated through a common pathway mediated by protein C. *Thromb Haemost* 66: 160-165.

Espinola-Klein C, Rupprecht HJ, Blankenberg S, et al 2000. Are morphological or functional changes in the carotid artery wall associated with *Chlamydia pneumoniae*, *Helicobacter pylori*, cytomegalovirus, or herpes simplex virus infection? *Stroke* 31:2127-2133.

Espinola-Klein C, Rupprecht HJ, Blankenberg S, Bickel C, Kopp Helmuth, Rippin G, Victor A, Hafner G, Schlumberger W, Meyer J, for the AtheroGene Investigators 2002(a). Impact of infectious burden on extent and long-term prognosis of atherosclerosis. *Circulation* 105:15-21.

Espinola-Klein C, Rupprecht H-J, Blankenberg S, Bickel C, Kopp H, Victor A, Hafner G, Prellwitz W, Schlumberger W, Meyer J 2002(b). Impact of infectious burden on progression of carotid atherosclerosis. *Stroke* 33:2581-2586.

Etminan M, Carleton B, Delaney JA, Padwal R 2004. Macrolide therapy for *Chlamydia pneumoniae* in the secondary prevention of coronary artery disease: a meta-analysis of randomized controlled trials. *Pharmacotherapy* 24:338-343.

Ezzahiri R, Nelissen-Vrancken HJMG, Kurvers HAJM, Stassen FRM, Vliegen I, Grauls GELM, van Pul MML, Kitslaar PJEHM, Bruggeman CA 2002. *Chlamydia pneumoniae* (*Chlamydia pneumoniae*) accelerates the formation of complex atherosclerotic lesions in Apo E3-Leiden mice. *Cardiovascular Research* 56:269-276.

Fagerberg B, Gnarpe J, Gnarpe H, Agewall S, Wikstrand J, 1999. *Chlamydia pneumoniae* but not cytomegalovirus antibodies are associated with future risk of stroke and cardiovascular disease: A prospective study in middle-aged to elderly men with treated hypertension. *Stroke* 30:299-305.

Faggiotto A, Ross R, Harker L 1984. Studies of hypercholesterolaemia in the non-human primate. I. Changes that lead to fatty streak formation. *Arteriosclerosis* 4:323-40.

Familoni OB, Odusan O, Ogun SA 2006. The pattern and prognostic features of QT intervals and dispersion in patients with acute ischaemic stroke. *J Natl Med Assoc* 98:1758-1762.

Feinberg WM, Pearce LA, Hart RG, Cushman M, Cornell ES, Lip GY, Bovill EG 1999. Markers of thrombin and platelet activity in patients with atrial fibrillation: correlation with stroke among 1531 participants in the stroke prevention in atrial fibrillation III study. *Stroke* 30:2547-2553.

Fiehn NE, Larsen T, Christiansen N, Holmstrup P, Schroeder TV 2005. Identification of periodontal pathogens in atherosclerotic vessels. *J Periodontol* 76:731-736.

Fields BS, Benson RF, Besser RE 2002. *Legionella* and Legionnaires' disease: 25 years of investigation. *Clinical Microbiology Reviews* 15:506-526.

File TMJr, Tan JS, Plouffe JF 1998. The role of atypical pathogens: *Mycoplasma pneumoniae*, *Chlamydia pneumoniae*, and *Legionella pneumophila* in respiratory infection. *Infectious Diseases Clinics of North America* 12:569-592.

Fong IW, Chiu B, Viira E et al 1997. Rabbit models for *Chlamydia pneumoniae* infection. *J Clin Microbiol* 35:48-52.

Fong IW 2002. Infections and their role in atherosclerotic vascular disease. *JADA* 133:7S-13S.

Fourestie V, Douceron H, Brugieres P et al 1993. Neurotrichinosis: a cerebrovascular disease associated with myocardial injury and hypereosinophilia. *Brain* 116:603-616.

Fournier PE, Marrie TJ, Raoult D 1998. Diagnosis of Q fever. *Journal of Clinical Microbiology* 36:1823-1834.

Franzin L, Scramuzza F 1995. Prevalence of *Legionella pneumophila* serogroup 1 antibodies in blood donors. *Eur J Epidemiol* 11:475-478.

Fu M, Wong KS, Lam WWM, Wong GWK 1998. Middle cerebral artery occlusion after recent *Mycoplasma pneumoniae* infection. *J Neurol Sci* 157:113-115.

Fure B, Bruun Wyller T, Thommessen B 2006. Electrocardiographic and troponin T changes in acute ischaemic stroke. *J Intern Med* 259:592-597.

Galante A, Silvestrini M, Stanzione P et al 1992. Leucocyte aggregation in acute cerebrovascular disease. *Acta Neurol Scand* 86: 446-449.

Ganesan V, Savvy L, Chong WK, Kirkham FJ 1999. Conventional cerebral angiography in children with ischaemic stroke. *Pediatr Neurol* 20:38-42.

Garcia JH, Wagner S, Liu KF 1995. Neurological deficit and extent of neuronal necrosis attributable to middle cerebral artery occlusion in rats: statistical validation. *Stroke* 26: 627-634.

Gaydos CA, Summersgill JT, Sahney NN, Ramirez JA, Quinn TC 1996. Replication of *Chlamydia pneumoniae* in vitro in human macrophages, endothelial cells, and aortic artery smooth muscle cells. *Infect Immun* 64:1614-1620.

Geist LJ, Monick MM, Stinski MF, Hunninghake GW 1991. The immediate early genes of human cytomegalovirus upregulate expression of the interleukin 2 and interleukin 2 receptor genes. *Am J Respir Cell Mol Biol* 5:292-296.

Geist LJ, Monick MM, Stinski MF, Hunninghake GW 1994. The immediate early genes of human cytomegalovirus upregulate tumor necrosis factor-alpha gene expression. *J Clin Invest* 93:474-478.

Gerrity RG 1981. The role of the monocyte in atherogenesis: I. Transition of blood-borne monocytes into foam cells in fatty lesions. *Am J Pathol* 103:181-190.

Ghosh K, Clements GB 1992. Surveillance of *Mycoplasma pneumoniae* infections in Scotland 1986-1991. *J infect* 25:221-227.

Gieffers J, Fullgraf H, Jahn J, Klinger M, Dalhoff K, Katus HA, Solbach W, Maass M 2001. *Chlamydia pneumoniae* infection in circulating human monocytes is refractory to antibiotic treatment. *Circulation* 103:351-356.

Gigateway, Association for Geographic Information, London, UK. Available at: <http://www.gigateway.org.uk/areasearch/default.html>

Gilbert A, Lion G 1889. Arterites infectieuses experimentales. *Comptes Rendus Hebdomadaires des Seances et Memoires de la Societe de Biologie* 6:583-600.



Gillum RF, Mussolino ME 2003. Education, poverty, and stroke incidence in whites and blacks: the NHANES I epidemiologic follow-up study. *J Clin Epidemiol* 56:188-195.

Gimenez-Sanchez F, Butler JC, Jernigan DB et al 2001. Treating cardiovascular disease with antimicrobial agents: a survey of knowledge, attitudes, and practices among physicians in the United States. *Clin Infect Dis* 33:171-6.

Glader CA, Stegmayr B, Boman J, Stenlund H, Weinehall L, Hallmans G, Dahlen G 1999. *Chlamydia pneumoniae* antibodies and high lipoprotein(a) levels do not predict ischaemic cerebral infarctions. Results from a nested case-control study in Northern Sweden. *Stroke* 30:2013-2018.

Glagov S, Zarins CK, Giddens DP et al 1988. Haemodynamics and atherosclerosis. Insights and perspectives gained from studies of human arteries. *Arch Pathol Lab Med* 112:1018-1031.

Gois J, Higuchi M, Reis M, Diamant J, Sousa J, Ramires J, Oliveira S 2006. Infectious agents, inflammation, and growth factors: how do they interact in the progression or stabilization of mild human atherosclerotic lesions? *Ann Vasc Surg* 20:638-645.

Goldstein DS 1979. The electrocardiogram in stroke: relationship to pathophysiological type and comparison with prior tracings. *Stroke* 10:253-259.

Goyal P, Kale SC, Chaudhry R, Chauhan S, Shah N 2007. Association of common chronic infections with coronary artery disease in patients without any conventional risk factors. *Indian J Med Res* 125:129-136.

Graham DY, Malaty HM, Evans DG, Evan DJ Jr, Klein PD, Adam E 1991. Epidemiology of *Helicobacter pylori* in an asymptomatic population in the United States. Effect of age, race, and socioeconomic status. *Gastroenterology* 100:1495-1501.

Grau AJ, Becher H, Ziegler CM et al 2004. Periodontal disease as a risk factor for ischemic stroke. *Stroke* 35:496-501.

Grau AJ, Bugge F, Becher H, Zimmermann E, Spiel M, Fent T, Maiwald M, Werle E, Zorn M, Hengel H, Hacke W 1998. Recent bacterial and viral infection is a risk factor for cerebral ischemia: clinical and biochemical studies. *Neurology* 50:196-203.

Grau AJ, Bugge F, Heindl S, Steichen-Wiehn C, Banerjee T, Maiwald M, Rohlf M, Suhr H, Fiehn W, Becher H, Hacke W 1995. Recent infection as a risk factor for cerebrovascular ischaemia. *Stroke* 26:373-379.

Grau AJ, Bugge F, Lichy C, Brandt T, Becher H, Rudi J 2001. *Helicobacter pylori* infection as an independent risk factor for cerebral ischaemia of atherothrombotic origin. *J Neurol Sci* 186:1-5.

Grau AJ, Fischer B, Barth C, Ling P, Lichy C, Bugge F 2005. Influenza vaccination is associated with a reduced risk of stroke. *Stroke* 36:1501-1506.

Grau AJ, Marquardt L, Lichy C 2006. The effect of infections and vaccinations on stroke risk. *Expert Rev Neurotherapeutics* 6:175-183.

Grayston JT 2003. Antibiotic treatment of atherosclerotic cardiovascular disease. *Circulation* 107:1228.

Grayston JT 2000. Background and current knowledge of *Chlamydia pneumoniae* and atherosclerosis. *J Infect Dis* 181:(Suppl 3), S402-S410.

Grayston JT, Kronmal RA, Jackson LA, Parisi AF, Muhlestein JB, Cohen JD, Rogers WJ, Crouse JR, Borrowdale SL, Schron E, Knirsch C, for the ACES Investigators 2005. Azithromycin for the secondary prevention of coronary events. *N Engl J Med* 352:1637-1645.

Greco F, Castellano Chiodo D, Sorge A, Perrini S, Sorge G 2006. Multiple arterial ischemic strokes in a child with moyamoya disease and *Mycoplasma pneumoniae* infection. *Minerva Pediatr* 58:63-68.

Greenlee JE, Rose JW. Controversies in neurological infectious diseases 2000. *Seminars in Neurology* 20:375-386.

Grundy E, Holt G 2001. The socioeconomic status of older adults: how should we measure it in studies of health inequalities? *J Epidemiol Community Health* 55:895-904.

Guallar-Castillon P, Artalejo FR, Banegas JRB, et al 1999. Factores ambientales en la vida temprana y nivel socioeconómico en la actualidad. *Med Clin (Barc)* 113 :444-446.

Gupta S 1999. *Chlamydia pneumoniae*, monocyte activation and antimicrobial therapy in coronary heart disease. MD Thesis, University of London, London.

Gupta S, Leatham EW, Carrington D et al 1997. Elevated *Chlamydia pneumoniae* antibodies, cardiovascular events and azithromycin in male survivors of myocardial infarction. *Circulation* 96: 404-417.

Gurfinkel E, Bozovich G, Daroca A et al 1997. Randomised trial of roxithromycin in non-Q-wave coronary syndromes: ROXIS pilot study. *Lancet* 350: 404-417.

Gurfinkel EP, Rozlosnik J, Bozovich G, Duronto E, Santos AD, Mautner B 1997. IgG antibodies to chlamydial and mycoplasma infection plus C-reactive protein related to poor outcome in unstable angina. *Arch Inst Cardiol Mex* 67:462-468.

Gutierrez J, Mendoza J, Fernandez F, Linares-Palomino J, Soto MJ, Maroto MC 2002. ELISA test to detect *Chlamydia pneumoniae* IgG. *J Basic Microbiol* 42:13-18.

Hachinski V 1998. Foreword. *Stroke*. *Lancet* 352: (suppl III) 1-30.

Hagiwara N, Toyoda K, Inoue T, Shimada H, Ibayashi S, Iida M, Okada Y 2007. Lack of association between infectious burden and carotid atherosclerosis in Japanese patients. *J Stroke Cerebrovasc Dis* 16:145-152.

Haider AW, Wilson PW, Larson MG, Evans JC, Michelson EL, Wolf PA, O'Donnell CJ, Levy D 2002. The association of seropositivity to *Helicobacter pylori*, *Chlamydia pneumoniae*, and *Cytomegalovirus* with risk of cardiovascular disease: a prospective study. *J Am Coll Cardiol* 40:1408-1413.

Hammerschlag MR 2000. *Chlamydia pneumoniae* and the lung. *Eur Resp J* 16:1001-1007.

Hand RA, Chandler AB 1962. Atherosclerosis metamorphosis of autologous pulmonary thromboemboli in the rabbit. *Am J Pathol* 40:469-486.

Hann M, Kaplan G, Camacho T 1987. Poverty and health: prospective evidence from the Alameda County study. *Am J Epidemiol* 125:989-998.

Hansen S, Als-Nielsen B, Damgaard M, Helø OH, Petersen L, Jespersen CM 2001. Intervention with clarithromycin in patients with stable coronary heart disease. *Heart Drug* 1:14-19.

Hansson GK, Hellstrand M, Rymo L et al 1989(a). Interferon gamma inhibits both proliferation and expression of differentiation – specific alpha-smooth muscle actin in arterial smooth muscle cells. *J Exp Med* 170:1595-1608.

Hansson GK, Holm J, Jonasson L 1989(b). Detection of activated T lymphocytes in the human atherosclerotic plaque. *Am J Pathol* 135:169-175.

Hart CL, Hole DJ, Davey Smith G 2000. Influence of socioeconomic circumstances in early and later life on stroke risk among men in a Scottish cohort study. *Stroke* 31:2093-2097.

Hart RG, Foster JW, Luther MF, Kanter MC 1990. Stroke in infective endocarditis. *Stroke* 21: 695-700.

Hauksdottir GS, Jonsson T, Sigurdardottir V, Löve A 1998. Seroepidemiology of *Mycoplasma pneumoniae* infections in Iceland 1987-96. *Scand J Infect Dis* 30:177-180.

Hauss WH 1962. Changes in metabolism of connective tissue associated with ageing and arterio- or atherosclerosis. *J Atherosclerosis Res* 6:50.

Hawker JI, Olowokure B, Sufi F, Weinberg J, Gill N, Wilson RC 2003. Social deprivation and hospital admission for respiratory infection: an ecological study. *Respiratory Medicine* 97:1219-1224.

Helms CM, Renner ED, Viner JP, Hierholzer Jr WJ, Wintermeyer LA, Johnson W 1980. Indirect immunofluorescence antibodies to *Legionella pneumophila*: frequency in a rural community. *J Clin Microbiol* 12:326-328.

Hendrix MG, Salimans MM, van Boven CP, Bruggeman CA 1990. High prevalence of latently present cytomegalovirus in arterial walls of patients suffering from grade III atherosclerosis. *Am J Pathol* 136:23-28.

Heng BH, Goh KT, Ng DL, Ling AE 1997. Surveillance of legionellosis and *Legionella* bacteria in the built environment in Singapore. *Ann Acad Med Singapore* 26:557-565.

Hesselvik JF, Malm J, Dahlback B, Blomback M 1991. Protein C, protein S and C4b-binding protein in severe infection and septic shock. *Thromb Haemost* 65: 126-129.

Heuschmann P, Neureiter D, Gesslein M, Craiovan B, Maass M, Faller G, Beck G, Neundoerfer B, Kolominsky-Rabas P 2001. Association between infection with *Helicobacter pylori* and *Chlamydia pneumoniae* and risk of ischaemic stroke subtypes: Results from a population-based case-control study. *Stroke* 32:2253-2258.

Higgins JP 2003. *Chlamydia pneumoniae* and coronary artery disease: the antibiotic trials. Mayo Clin Proc 78:321-332.

Higuchi ML, Gois JM, Reis MM, Higuchi-Dos-Santos MH, Diament J, Sousa JM, Ramires JA, Oliveira SA 2006. Co-infection ratios versus inflammation, growth factors and progression of early atheromas. APMIS 114:338-344.

Higuchi ML, Ramires JAF 2002. Infectious agents in coronary atheromas: a possible role in the pathogenesis of plaque rupture and acute myocardial infarction. Rev Inst Med trop S Paulo 44:217-224.

Higuchi ML, Reis MM, Sambiasi NV, Palomino SAP, Castelli JB, Gutierrez PS, Aiello VD, Ramires JAF 2003. Coinfection with *Mycoplasma pneumoniae* and *Chlamydia pneumoniae* in ruptured plaques associated with acute myocardial infarction. Arq Bras Cardiol 81:12-22.

Higuchi ML, Sambiasi N, Palomino S, Gutierrez P, Demarchi LM, Aiello VD, Ramires JAF 2000. Detection of *Mycoplasma pneumoniae* and *Chlamydia pneumoniae* in ruptured atherosclerotic plaques. Braz J Med Biol Res 33:1023-1026.

Hillis GS, Pearson CV, Harding SA, Sutherland S, Ludlam CA, Marioni JC, Prescott RJ, Fox KA, Flapan AD 2004. Effects of a brief course of azithromycin on soluble cell adhesion molecules and markers of inflammation in survivors of an acute coronary syndrome: a double-blind, randomised, placebo-controlled study. Am Heart J 148:72-79.

Hindiyeh M, Carroll KC 2000. Laboratory diagnosis of atypical pneumonia. Seminars in Respiratory Infections 15:101-113.

Hingorani AD, Cross J, Kharbada RK et al 2000. Acute systemic inflammation impairs endothelium dependent dilatation in humans. Circulation 102:994-999.

Horne BD, Muhlestein JB, Carlquist JF, Ohana B, Lipson M, Kahn N, Habashi J, Bair TL, Anderson JL 2000. IgA seropositivity to *Mycoplasma pneumoniae* predicts the diagnosis of coronary artery disease. JACC 35(suppl A):312A.

Hoymans VY, Bosmans JM, Van Renterghem L, Mak R, Ursi D, Wuyts F, Vrints CJ, Ieven M 2003. Importance of methodology in determination of *Chlamydia pneumoniae* seropositivity in healthy subjects and in patients with coronary atherosclerosis. J Clin Microbiol 41:4049-4053.

Hu H, Pierce GN, Zhong G 1999. The atherogenic effects of chlamydia are dependent on serum cholesterol and specific to *Chlamydia pneumoniae*. J Clin Invest 103:747-753.

Hujoel PP, Drangsholt M, Spiekerman C, DeRouen TA 2000. Periodontal disease and coronary heart disease risk. JAMA 284:1406-1410.

Ieven MM, Hoymans VY 2005. Involvement of *Chlamydia pneumoniae* in atherosclerosis: more evidence for lack of evidence. Journal of Clinical Microbiology 43:19-24.

Igarashi M, Gilmartin RC, Gerald B et al 1984. Cerebral arteritis and bacterial meningitis. Arch Neurol 41: 531-535.

International Labour Office, 1990. ISCO-88: International Standard Classification of Occupations. Geneva: ILO.

Ishizaka N, Ishizaka Y, Takahashi E, Toda E, Hashimoto H, Nagai R, Yamakado M 2002(a). Association between hepatitis C virus seropositivity, carotid-artery plaque, and intima-media thickening. Lancet 359:133-135.

Ishizaka N, Ishizaka Y, Takahashi E, Toda E, Hashimoto H, Ohno M, Nagai R, Yamakado M 2002(b). Increased prevalence of carotid atherosclerosis in hepatitis B virus carriers. Circulation 105:1028-1030.

Jabaudon D, Sztajzel J, Sievert K, Landis T, Sztajzel R 2004. Usefulness of ambulatory 7-day ECG monitoring for the detection of atrial fibrillation and flutter after acute stroke and transient ischaemic attack. *Stroke* 35:1647-1651.

Jackson LA, Campbell LA, Schmidt RA et al 1997. Specificity of detection of *Chlamydia pneumoniae* in cardiovascular atheroma- evaluation of the innocent bystander hypothesis. *Am J Pathol* 150: 1785-1790.

Jackson LA, Smith NL, Heckbert SR et al 2000. Past use of erythromycin, tetracycline, or doxycycline is not associated with risk of first myocardial infarction. *J Infect Dis* 181:S563-5.

Jakovljevic D, Sarti C, Sivenius J, Torppa J, Mahonen M, Immonen-Raiha P, Kaarsalo E, Alhainen K, Kuulasmaa K, Tuomilehto J, Puska P, Salomaa V 2001. Socioeconomic status and ischaemic stroke- the FINMONICA stroke register. *Stroke* 32:1492-1498.

Jarman B 1983. Identification of underprivileged areas. *BMJ* 286:1705-1709.

Jarman B 1984. Underprivileged areas: validation and distribution of scores. *BMJ* 289:1587-1592.

Jespersen CM, Als-Nielsen B, Damgaard M, Hansen JF, Hansen S, Helo OH, Hildebrandt P, Hilden J, Jensen GB, Kastrup J, Kolmos HJ, Kjoller E, Lind I, Nielsen H, Petersen L, Gluud C; CLARICOR Trial Group 2006. *BMJ* 21:332:22-27.

Johnsen SP, Overvad K, Ostergaard L, Tjonneland A, Husted SE, Sorensen HT 2005. *Chlamydia pneumoniae* seropositivity and risk of ischemic stroke: a nested case-control study. *European Journal of Epidemiology* 20:59-65.

Johnson JD, Raff MJ, Van Arsdall JA 1984. Neurologic manifestations of Legionnaires' disease. *Medicine* 63:303-310.



Johnston SC, Messina LM, Browner WS, Lawton MT, Morris C, Dean D 2001. C-reactive protein levels and viable *Chlamydia pneumoniae* in carotid artery atherosclerosis. *Stroke* 32:2748-2752.

Jones HR Jr, Siekert RG, Geraci JE 1969. Neurologic manifestations of bacterial endocarditis. *Ann Intern Med* 71: 21-28.

Jordan R, Verlander N, Olowokure B, Hawker JI 2006. Age, sex, material deprivation and respiratory mortality. *Resp Med* 100:1282-1285.

Jurgens G, Hoff HF, Chisolm GM et al 1987. Modification of human serum low density lipoprotein by oxidation – characterisation and pathophysiological implications. *Chem Phys Lipids* 45:315-336.

Kalayoglu MV, Byrne GI 1998. Induction of macrophage foam cell formation by *Chlamydia pneumoniae*. *The Journal of Infectious Diseases* 177:725-729.

Kalayoglu MV, Hoerneman B, La Verda D et al 1999. Cellular oxidation of low-density lipoprotein by *Chlamydia pneumoniae*. *J Infect Dis* 180:780-790.

Kalayoglu MV, Libby P, Byrne GI 2002. *Chlamydia pneumoniae* as an emerging risk factor in cardiovascular disease. *JAMA* 288:2724-2731.

Kalra L 1998. Stroke: Clinical presentation and management. In: Tallis RC, Fillit HM, Brocklehurst JC (5<sup>th</sup> edition). *Textbook of Geriatric Medicine and Gerontology*, 499-532. Churchill Livingstone.

Kanamoto Y, Ouchi K, Mizui M, Ushio M, Usui T 1991. Prevalence of antibody to *Chlamydia pneumoniae* TWAR in Japan. *J Clin Microbiol* 29:816-818.

Kaplan GA, Keil JE 1993. Socioeconomic factors and cardiovascular disease: a review of the literature. *Circulation* 88:1973-1998.

Kapral MK, Wang H, Mamdani M, Tu JV 2002. Effect of socioeconomic status on treatment and mortality after stroke. *Stroke* 33:268-275.

Kaukoranta-Tolvanen SSE, Teppo AM, Laitinen K, Saikku P, Linnavuori K, Leinonen M 1996. Growth of *Chlamydia pneumoniae* in cultured human peripheral blood mononuclear cells and induction of a cytokine response. *Microb Pathog* 21:215-221.

Kawamoto R, Kajiwarra T, Oka Y, Takagi Y 2003. An association between an antibody against *Chlamydia pneumoniae* and ischaemic stroke in elderly Japanese. *Intern Med* 42:571-575.

Kawashima N, Kawada J 2000. *Chlamydia pneumoniae* antibody titres in patients with acute ischaemic stroke. (in Japanese). *Rinsho Shinkeigaku - Clinical Neurology* 40:1063-1068.

Khairy P, Rinfret S, Tardif JC, Marchand R, Shapiro S, Brophy J, Dupuis J 2003. Absence of association between infectious agents and endothelial function in healthy young men. *Circulation* 107:1966-1971.

Khechinashvili G, Asplund K 2002. Electrocardiographic changes in patients with acute stroke: a systematic review. *Cerebrovasc Dis* 14:67-76.

Kiebertz KD, Eskin TA, Ketonen L, Tuite MJ 1993. Opportunistic cerebral vasculopathy and stroke in patients with the acquired immunodeficiency syndrome. *Arch Neurol* 50: 430-432.

Kiechl S, Egger G, Mayr M, Wiedermann CJ, Bonora E, Oberhollenzer F, Muggeo M, Xu Qingbo, Wick G, Poewe W, Willeit J 2001. Chronic infections and the risk of carotid atherosclerosis: prospective results from a large population study. *Circulation* 103:1064-1070.

Kim WJ, Hahn TW, Kim DY, Lee MG, Jung KS, Ogawa M, Kishimoto T, Lee ME, Lee SJ 2006. Seroprevalence of *Coxiella burnetii* infection in dairy cattle and non-

symptomatic people for routine health screening in Korea. J Korean Med Sci 21:823-826.

Ko WC, Liang CC, Chen HY, Chuang YC 2000. Seroprevalence of *Coxiella burnetii* infection in Southern Taiwan. J Formos Med Assoc 99:33-38.

Koenig W 1999. Atherosclerosis involves more than just lipids: focus on inflammation. Eur Heart J Supplements 1(Suppl T):19-26.

Koenig W, Rothenbacher D, Hoffmeister A et al 1999. Infection with *Helicobacter pylori* is not a major independent risk factor for stable coronary heart disease. Circulation 100:2326-2331.

Kol A, Sukhova GK, Lichtman AH, Libby P 1998. Chlamydia heat shock protein 60 localises in human atheroma and regulates macrophage tumour necrosis factor- $\alpha$  and matrix metalloproteinase expression. Circulation 98:300-307.

Koster A, Bosma H, Penninx BWJH, Newman AB, Harris TB, van Eijk JTM, Kempen GIJM, Simonsick EM, Johnson KC, Rooks RN, Ayonayon HN, Rubin SM, Kritchevsky SB, for the Health ABC Study 2006. Association of inflammatory markers with socioeconomic status. J Gerontol A Biol Sci Med Sci 61:284-290.

Kovacova E, Kazar J 2002. Q fever- still a query and underestimated infectious disease. Acta Virologica 46:193-210.

Kowalik TF, Wing B, Haskill JS, Azizkhan JC, Baldwin AS, Huang ES 1993. Multiple mechanisms are implicated in the regulation of NF-B activity during human cytomegalovirus infection. Proc Natl Acad Sci USA 90:1107-1111.

Ku DN, Giddens DP, Zarins CK et al 1985. Pulsatile flow and atherosclerosis in human carotid bifurcation. Positive correlation between plaque location and low oscillating shear stress. Arteriosclerosis 5:293-302.

- Kurth T, Berger K 2007. The socioeconomic stroke puzzle. *Stroke* 38:4-5.
- Laitinen K, Laurila A, Pyhala L et al 1997. *Chlamydia pneumoniae* induces inflammatory changes in the aortas of rabbits. *Infect Immun* 65:4832-5.
- Lavallee P, Perchaud V, Gautier-Bertrand M, Grabli D, Amarenco P 2002. Association between influenza vaccination and reduced risk of brain infarction. *Stroke* 33:513-518.
- Lazar J, Manzella S, Moonjelly J, Wirkowski E, Cohen TJ 2003. The prognostic value of QT dispersion in patients presenting with acute neurological events. *Journal of Invasive Cardiology* 15:31-35.
- Leinonen M 1993. Pathogenetic mechanisms and epidemiology of *Chlamydia pneumoniae*. *Eur Heart J* 14(suppl K):57-61.
- Leinonen M, Saikku P 2002. Evidence for infectious agents in cardiovascular disease and atherosclerosis. *Lancet Infectious Diseases* 2:11-17.
- Lemstrom KB, Aho PT, Bruggeman CA, Hayry PJ 1994. Cytomegalovirus infection enhances mRNA expression of platelet-derived growth factor-BB and transforming growth factor-beta 1 in rat aortic allografts: possible mechanism for cytomegalovirus-enhanced graft arteriosclerosis. *Arterioscler Thromb* 14:2043-2052.
- Leon DA, Davey Smith G 2000. Infant mortality, stomach cancer, stroke and coronary heart disease: ecological analysis. *BMJ* 320:1705-1706.
- Leonardi S, Pavone P, Rotolo N, La Rosa M 2005. Stroke in two children with *Mycoplasma pneumoniae* infection. A causal or casual relationship? *Pediatr Infect Dis* 24:843-845.
- Leopold NA 1993. Chickenpox stroke in an adult. *Neurology* 43: 1852-1853.

Leowattana W, Bhuripanyo K, Singhaviranon L et al 2001. Roxithromycin in prevention of acute coronary syndrome associated with *Chlamydia pneumoniae* infection: a randomised placebo controlled trial. *J Med Assoc Thai* 84:S669-S675.

Li L, Messas E, Batista EL Jr, Levine RA, Amar S 2002. *Porphyromonas gingivalis* infection accelerates the progression of atherosclerosis in a heterozygous apolipoprotein E-deficient murine model. *Circulation* 105:861-867.

Lieberman D, Lieberman D, Ben-Yaakov M, Shmarkov O, Gelfer Y, Varshavsky R, Ohana B, Lazarovich Z, Boldur I 2002. Serological evidence of *Mycoplasma pneumoniae* infection in acute exacerbation of COPD. *Diagn Microbiol Infect Dis* 44:1-6.

Lin HJ, Wolf PA, Kelly-Hayes M, Beiser AS, Kase CS, Benjamin EJ, D'Agostino RB 1996. Stroke severity in atrial fibrillation. The Framingham Study. *Stroke* 27:1760-1764.

Lind K, Benzon MW, Skov Jensen J, Clyde Jr. WA 1997. A seroepidemiological study of *Mycoplasma pneumoniae* infections in Denmark over the 50-year period 1946-1995. *Eur J Epidemiol* 13:581-586.

Lindgren A, Wohlfart B, Pahlm O, Johansson BB 1994. Electrocardiographic changes in stroke patients without primary heart disease. *Clinical Physiology* 14:223-231.

Linnanmaki E, Leinonen M, Ekman MR et al 1993. *Chlamydia pneumoniae* specific circulating immune complexes in chronic coronary heart disease. *Circulation* 87:1130-1134.

Linsberg PJ, Grau AJ 2003. Inflammation and infections as risk factors for ischemic stroke. *Stroke* 34:2518-2532.

Liuba P, Pesonen E, Paakkari I, Forslid A, Sandstrom S 2002. Acute *Chlamydia pneumoniae* infection causes diffuse coronary endothelial dysfunction in pigs. *JACC* 39(suppl 2):S277.

Lobos T, Moreno R, Carstens M, Domenech A, Piemonte P, Juliet C, Pfenninger A 1993. Seroprevalence of *Legionella pneumophila* infection in healthy, adults from Santiago, Chile (in Spanish). Rev Med Chil 121:1123-1127.

Lobzin IuV, Boitsov SA, Filippov AE, Linchak RM, Mangutov DA 2005. Effect of respiratory infections on the clinical course of coronary artery disease. (in Russian) Klin Med (Mosk) 83:22-26.

Loftus IM, Naylor AR, Bell PRF, Thompson MM 2002. Matrix metalloproteinases and atherosclerotic plaque instability. British Journal of Surgery 89:680-694.

Lovey P-Y, Morabia A, Bleed D, Peter O, Dupuis G, Petite J 1999. Long term vascular complications of *Coxiella burnetii* infection in Switzerland: cohort study. British Medical Journal 319:284-286.

Luchsinger JA, Pablos-Mendez A, Knirsch C, Rabinowitz D, Shea S 2001. Antibiotic use and risk of ischaemic stroke in the elderly. Am J Med 111:361-366.

Luchsinger JA, Pablos-Méndez A, Knirsch C, Rabinowitz D, Shea S 2002. Relation of antibiotic use to risk of myocardial infarction in the general population. Am J Cardiol 89:18-21.

Lynn DJ, Kane JG, Parker RH 1977. Haemophilus parainfluenzae and influenzae endocarditis: a review of forty cases. Medicine 56:115-128.

Macko RF, Ameriso SF, Barndt R et al 1996. Precipitants of brain infarction: roles of preceding infection / inflammation and recent psychological stress. Stroke 27:1999-2004.

Macko RF, Ameriso SF, Gruber A et al 1996(b). Impairments of the protein C system and fibrinolysis in infection-associated stroke. Stroke 27:2005-2011.

Malmgren R, Bamford J, Warlow C et al 1989. Projecting the number of patients with first-ever strokes and patients newly handicapped by stroke in England and Wales. *BMJ* 298:656-660.

Maraha B, Berg H, Scheffer GJ, van der Zee A, Bergmans A, Misere J, Kluytmans J, Peeters M 2001. Correlation between detection methods of *Chlamydia pneumoniae* in atherosclerotic and non-atherosclerotic tissues. *Diagn Microbiol Infect Dis* 39:139-143.

Maraha B, den Heijer M, Kluytmans J, Peeters MF 2004. Impact of serological methodology on assessment of the link between *Chlamydia pneumoniae* and vascular diseases. *Clin Diagn Lab Immunol* 11:789-791.

Maraha B, van der Zee A, Bergmans AMC, Pan ML, Peeters MF, Berg HF, Scheffer GJ, Kluytmans JAJW 2000. Is *Mycoplasma pneumoniae* associated with vascular disease? *J Clin Microbiol* 38:935-936.

Marcus AJ, Safier LB, Ullman HL et al 1988. Platelet-neutrophil interactions. *J Biol Chem* 263: 2223-2229.

Marini C, De Santis F, Sacco S, Russo T, Olivieri L, Totaro R, Carolei A 2005. Contribution of atrial fibrillation to incidence and outcome of ischaemic stroke: results from a population-based study. *Stroke* 36:1115-1119.

Markus HS, Mendall MA 1998. *Helicobacter pylori* infection: a risk factor for ischaemic cerebrovascular disease and carotid atheroma. *J Neurol Neurosurg Psychiatry* 64:104-107.

Marrie TJ, Peeling RW, Fine MJ, Singer DE, Coley CM, Kapoor WN 1996. Ambulatory patients with community-acquired pneumonia: the frequency of atypical agents and clinical course. *Am J Med* 101:508-515.

Marrie TJ, Pollak PT 1995. Seroepidemiology of Q fever in Nova Scotia: evidence for age dependent cohorts and geographical distribution. *Eur J Epidemiol* 11:47-54.

Marrie TJ, Raoult D 2002. Update on Q fever, including Q fever endocarditis. *Curr Clin Top Infect Dis* 22:97-124.

Masuda J, Ross R 1990. Atherogenesis during low level hypercholesterolaemia in the non-human primate. I. Fatty streak formation. *Arteriosclerosis* 10:164-177.

Mattila KJ, Valtonen VV, Nieminen MS, Asikainen S 1998. Role of infection as a risk factor for atherosclerosis, myocardial infarction, and stroke. *Clin Infect Dis* 26:719-734.

Maurin M, Raoult D 1999. Q fever. *Clinical Microbiology Reviews*. 12:518-553.

Mayr M, Metzler B, Kiechl S, Willeit J, Schett G, Xu Q, Wick G 1999. Endothelial cytotoxicity mediated by serum antibodies to heat shock proteins of *Escherichia coli* and *Chlamydia pneumoniae*: immune reactions to heat shock proteins as a possible link between infection and atherosclerosis. *Circulation* 99:1560-1566.

McDermott MM, Lefevre F, Arron M, Martin GJ, Biller J 1994. ST segment depression detected by continuous electrocardiography in patients with acute ischaemic stroke or transient ischaemic attack. *Stroke* 25:1820-1824.

McGill HC Jr 1968. Fatty streaks in the coronary arteries and aorta. *Lab Invest* 18:560-564.

McNeil HP, Chesterman CN, Krilis SA 1991. Immunology and clinical importance of antiphospholipid antibodies. *Adv in Immunol* 49: 193-280.

Meier CR, Derby LE, Jick SS et al 1999. Antibiotics and risk of subsequent first-time acute myocardial infarction. *JAMA* 281:427-31.

Melissano G, Blasi F, Esposito G, Tarsia P, Dordoni L, Arosio C, Tshomba Y, Fagetti L, Allegra L, Chiesa R 1999. *Chlamydia pneumoniae* eradication from carotid plaques. Results of an open randomized treatment study. *Eur J Vas Endovasc Surg* 18:355-359.



Melnick JL, Adam E, Debakey ME 1990. Possible role of cytomegalovirus in atherogenesis. JAMA 263:2204-2207.

Mendall MA, Goggin PM, Molineaux N et al 1992. Childhood living conditions and *Helicobacter pylori* seropositivity in adult life. Lancet 339:896-897.

Messmer TO, Martinez J, Hassouna F, Zell ER, Harris W, Dowell S, Carlone GM 2001. Comparison of two commercial microimmunofluorescence kits and an enzyme immunoassay kit for detection of serum immunoglobulin G antibodies to *Chlamydia pneumoniae*. Clinical and Diagnostic Laboratory Immunology 8:588-592.

Miller SA, Selzman CH, Shames BD et al 2000. *Chlamydia pneumoniae* activates nuclear factor  $\kappa$ B and activator protein 1 in human vascular smooth muscle and induces cellular proliferation. J Surg Res 90:76-81.

Molaschi M, Ponzetto M, Romin R, Berrino E, Fabris F 1995. Changes in the electrocardiogram in the elderly patient. The limits between normality and pathology. (Article in Italian) Recenti Prog Med 86:32-36.

Molestina RE, Dean D, Miller RD et al 1998. Characterisation of a strain of *Chlamydia pneumoniae* isolated from a coronary atheroma by analysis of the omp 1 gene and biological activity in human endothelial cells. Infect Immun 66:1370-1376.

Molestina RE, Miller RD, Lentsch AB et al 2000. Requirement for NF-kappaB in transcriptional activation of monocyte chemotactic protein 1 by *Chlamydia pneumoniae* in human endothelial cells. Infect Immun 68:4282-8

Molestina RE, Miller RD, Ramirez JA et al 1999. Infection of human endothelial cells with *Chlamydia pneumoniae* stimulates transendothelial migration of neutrophils and monocytes. Infect Immun 67:1323-1330.

Momiyama Y, Ohmori R, Taniguchi H, Nakamura H, Ohsuzu F 2004. Association of *Mycoplasma pneumoniae* infection with coronary artery disease and its interaction with chlamydial infection. *Atherosclerosis* 176:139-144.

Mora R, Lupu F, Simionescu N 1987. Prelesional events in atherogenesis. Colonisation of apolipoprotein B, unesterised cholesterol and extracellular phospholipid liposomes in the aorta of hyperlipidaemic rabbit. *Atherosclerosis* 67: 143-154.

Mosorin M, Juvonen J, Biancari F et al 2001. Use of doxycycline to decrease the growth rate of abdominal aortic aneurysms: a randomized, double-blind, placebo-controlled pilot trial. *J Vas Surg* 34:606-610.

Muhlestein JB, Anderson JL, Carlquist JF et al 2000. Randomised secondary prevention trial of azithromycin in patients with CAD: primary clinical results of the ACADEMIC Study. *Circulation* 102:1755-1760.

Muhlestein JB, Horne BD, Carlquist JF, et al 2000. Cytomegalovirus seropositivity and C-reactive protein have independent and combined predictive value for mortality in patients with angiographically demonstrated coronary artery disease. *Circulation* 103:1917-1923.

Mulder LJMM, Spierings ELH 1987(a). Stroke due to intravascular coagulation in *Mycoplasma pneumoniae* infection. *Lancet* 2:1152-1153.

Mulder LJMM, Spierings ELH 1987(b). Stroke in a young adult with *Mycoplasma pneumoniae* infection complicated by intravascular coagulation. *Neurology* 37:1430-1431.

Munro JM, Cotran RS 1988. The pathogenesis of atherosclerosis: atherogenesis and inflammation. *Lab Invest* 58: 249-261.

Murdoch DR 2003. Diagnosis of Legionella infection. *Clinical Infectious Diseases* 36:64-69.

Murphy EA, Streblow DN, Nelson JA, Stinski MF, 2000. The human cytomegalovirus IE86 protein can block cell cycle progression after inducing transition into the S phase of permissive cells. J Virol 74:7108-7118.

Murray CJL, Lopez AD 1996. The global burden of disease: a comprehensive assessment of mortality and disability from diseases, injuries, and risk factors in 1990 and projected to 2020. Boston: Harvard University Press.

Murray CJL, Lopez AD 1997. Mortality by cause for eight regions of the world: global burden of disease study. Lancet 349: 1269-1276.

Myers MG, Norris JW, Hachinski VC, Weingert ME, Sole MJ 1982. Cardiac sequelae of acute stroke. Stroke 13:838-842.

Nadarajah M, Singam S, Jalil HA 1987. Sero-survey for Legionella pneumophila antibodies- Singapore experience. Ann Acad Med Singapore 16:583-585.

Nadareishvili ZG, Koziol DE, Szekely B et al 2001. Increased CD8+ T cells associated with *Chlamydia pneumoniae* in symptomatic carotid plaque. Stroke 32:1966-1972.

Nakahata C, Kittka E, Fujii H, Sakano T, Usui T 1983. A case of cerebral infarction associated with *Mycoplasma pneumoniae* infection. Hiroshima J Med Sci 32:277-279.

Nencini P, Sarti C, Innocenti R, Pracucci G, Inzitari D 2003. Acute inflammatory events and ischemic stroke subtypes. Cerebrovas Dis 15 :215-221.

Neumann F, Kastrati A, Miethke T et al 2001. Treatment of *Chlamydia pneumoniae* infection with roxithromycin and effect on neointima proliferation after coronary stent placement (ISAR-3): a randomised, double-blind, placebo-controlled trial. Lancet 357:2085-2089.

Ngeh J, Anand V, Gupta S 2002. *Chlamydia pneumoniae* and atherosclerosis – what we know and what we don't. Clinical Microbiology and Infection 8:2-13.

Ngeh J, Goodbourn C 2005(a). *Chlamydia pneumoniae*, *Mycoplasma pneumoniae*, and *Legionella pneumophila* in Elderly Patients with Stroke (C-PEPS, M-PEPS, L-PEPS): a case-control study on the infectious burden of atypical respiratory pathogens in elderly patients with acute cerebrovascular disease. *Stroke* 36:259-263.

Ngeh J, Goodbourn C 2005(b). *Coxiella burnetii* and atypical respiratory infectious burden in stroke. *Stroke* 36:1356

Ngeh J, Gupta S 2004. *Chlamydia pneumoniae* and atherosclerosis: an overview of the association. In: Herman Friedman, Yoshimasa Yamamoto, Mauro Bendinelli, eds. *Chlamydia pneumoniae* Infection and Disease. New York: Kluwer Academic / Plenum Publishers; 113-133.

Ngeh J, Gupta S 2000. *Chlamydia pneumoniae* infection and atherosclerotic vascular disease: causal or coincidental link? *ASM News* (American Society for Microbiology) 66:732-737.

Ngeh J, Gupta S 2002. Inflammation and infection in coronary artery disease. In: Jackson G, ed. *Cardiology: Current Perspectives*. London: Martin Dunitz Ltd.

Ngeh J, Gupta S 2001. Inflammation, infection and antimicrobial therapy in coronary heart disease - where do we currently stand? *Fundamental & Clinical Pharmacology* 15:85-93.

Ngeh J, Gupta S, Goodbourn C 2004(a). The reproducibility of an enzyme-linked immunosorbent assay for detection of *Chlamydia pneumoniae*-specific antibodies. *Clin Microbiol Infect* 10:171-174.

Ngeh J, Gupta S, Goodbourn C, McElligott G 2004(b). *Mycoplasma pneumoniae* in elderly patients with stroke: a case-control study on the seroprevalence of *Mycoplasma pneumoniae* in elderly patients with acute cerebrovascular disease - The M-PEPS Study. *Cerebrovasc Dis* 17:314-319.

Ngeh J, Gupta S, Goodbourn C, Panayiotou B, McElligott G 2003. *Chlamydia pneumoniae* in elderly patients with stroke (C-PEPS): a case-control study on the seroprevalence of *Chlamydia pneumoniae* in elderly patients with acute cerebrovascular disease. *Cerebrovascular Diseases* 15:11-16.

Ngeh J, Gupta S, Goodbourn C, Panayiotou B, McElligott G 2001. Seroprevalence of *Chlamydia pneumoniae* in elderly stroke and medical patients: a case-control study. *Age and Ageing* 30(suppl 2): S60.

Ngeh J, Hackshaw A, Gupta S 2007. The relationship between socioeconomic deprivation, atypical respiratory infections and survival outcome in elderly stroke and medical patients. *Cerebrovascular Diseases* 24:546-547.

Ngeh JKT 2004. A case-control study on the prevalence of electrocardiographic rhythms and ischaemic changes in elderly patients with acute cerebrovascular disease. *American Journal of Geriatric Cardiology* 13:248-251

Ngeh JKT 2000. *Chlamydia pneumoniae* in elderly patients with stroke study (CPEPS): a case-control study on the seroprevalence of *Chlamydia pneumoniae* in patients aged over 65 years admitted with acute stroke or transient ischaemic attack. MSc Dissertation, University of Keele, UK.

NHS Strategic Tracing Service 2005. NHS Connecting for Health, Department of Health, UK.

Available at: <http://www.connectingforhealth.nhs.uk/systemsandservices/nsts>

Nichol KL, Nordin J, Mullooly J, Lask R, Fillbrandt K, Iwane M 2003. Influenza vaccination and reduction in hospitalizations for cardiac disease and stroke among the elderly. *N Engl J Med* 348:1322-1332.

Nieto FJ 1998. Infections and atherosclerosis: new clues from old hypothesis? *Am J Epidemiol* 148(10): 937-948.

Nieto FJ, Adam E, Sorlie P et al 1996. Cohort study of cytomegalovirus infection as a risk factor for carotid intimal-medial thickening, a measure of subclinical atherosclerosis. *Circulation* 94:922-927.

Niu YH, Ge JB, Xu CF, Shi JH, Jin XJ, Qian JY, Zou YZ 2005. Impact of pathogen burden on in-stent restenosis in patients after coronary stent implantation. *Chin Med J (Engl)* 118:1786-1790.

Njamnshi AK, Blackett KN, Mbuagbaw JN, Gumedze F, Gupta S, Wiysonge CS 2006. Chronic *Chlamydia pneumoniae* infection and stroke in Cameroon: a case-control study. *Stroke* 37:796-799.

Noah ND 1976. Epidemiology of *Mycoplasma pneumoniae* infections in the United Kingdom: an analysis of report to the Public Health Laboratory Service of England and Wales. *Infection* 4(4) Suppl 1:25-28.

Noah ND, Urquhart AM 1980. Epidemiology of *Mycoplasma pneumoniae* infection in the British Isles, 1974-9. *J Infect* 2:191-194.

Numazaki K, Ikebe T, Chiba S 1996. Detection of serum antibodies against *Chlamydia pneumoniae* by ELISA. *FEMS Immunology and Medical Microbiology* 14: 179-83.

O'Brien KD 1994. The biology of the artery wall in atherogenesis. *Med Clin N Amer* 78:41-67.

O'Connor CM, Dunne MW, Pfeffer MA et al 2003. Azithromycin for the secondary prevention of coronary heart disease events: the WIZARD study: a randomised controlled trial. *JAMA* 290:1459-1466.

Ode B, Cronberg S 1976. Infection and intracranial arterial thrombosis. *Lancet* 2:863-864.

Office of Population Censuses and Surveys, 1991. Standard Occupational Classification. London: HMSO.

Oppenheimer S 2002. The heart of the matter. *Cerebrovasc Dis* 14:65-66.

Oppenheimer SM, Gelb AW, Girvin JP, Hachinski VC 1992. Cardiovascular effects of human insular stimulation. *Neurology* 42:1727-1732.

Oppenheimer SM, Hachinski VC 1992. The cardiac consequences of stroke. *Neurologic Clinics* 10:167-176.

Ossewaarde JM, Feskens EJ, De Vries A, Vallinga CE, Kromhout D 1998. *Chlamydia pneumoniae* is a risk factor for coronary heart disease in symptom-free elderly men, but *Helicobacter pylori* and cytomegalovirus are not. *Epidemiol Infect* 120:93-99.

Ossewaarde JM, Tuuminen T, Boersma WG, Sandstrom M, Palomaki P, Boman J 2000. A preliminary evaluation of a new enzyme immunoassay to detect *Chlamydia pneumoniae*-specific antibodies. *Journal of Microbiological Methods* 43: 117-25.

Ovetchkine P, Brugieres P, Seradj A, Reinert P, Cohen R 2002. An 8-y-old boy with acute stroke and radiological signs of cerebral vasculitis after recent *Mycoplasma pneumoniae* infection. *Scand J Infect Dis* 34:307-309.

Padovan CS, Pfister HW, Bense S, Fingerle V, Abele-Horn M 2001. Detection of *Mycoplasma pneumoniae* DNA in cerebrospinal fluid of a patient with *M. pneumoniae* infection-‘associated’ stroke. *Clin Infect Dis* e119-121.

Paganini-Hill A, Lozano E, Fischberg G, Perez Barreto M, Rajamani K, Ameriso SF, Heseltine PNR, Fisher M 2003. Infection and risk of ischemic stroke: differences among stroke subtypes. *Stroke* 34:452-457.

Palm F, Grau A 2007. Infection as a risk factor for stroke. *Future Neurol* 2:59-71.

Paltiel O, Kark JD, Leinonen M, Saikku P 1995. High prevalence of antibodies to *Chlamydia pneumoniae*: determinants of IgG and IgA seropositivity among Jerusalem residents. *Epidemiol Infect* 114:465-473.

Parchure N, Zouridakis EG, Kaski JC 2002. Effect of azithromycin treatment on endothelial function in patients with coronary artery disease and evidence of *Chlamydia pneumoniae* infection. *Circulation* 105:1298-1303.

Park YD, Belman AL, Kim T-S et al 1990. Stroke in pediatric acquired immunodeficiency syndrome. *Ann Neurol* 28: 303-311.

Parke AL, Weinstein RE, Bona RD et al 1992. The thrombotic diathesis associated with the presence of phospholipid antibodies may be due to low levels of free protein S. *Am J Med* 93: 49-56.

Parker P, Puck J, Fernandez F 1981. Cerebral infarction associated with *Mycoplasma pneumoniae*. *Paediatrics* 67:373-375.

Pasceri V, Cammarota G, Patti G et al 1998. Association of virulent *Helicobacter pylori* strains with ischemic heart disease. *Circulation* 97:1675-1679.

Pascual-Velasco F, Montes M, Marimon JM, Cilla G 1998. High seroprevalence of *Coxiella burnetii* infection in Eastern Cantabria (Spain). *Int J Epidemiol* 27:142-145.

Passbender K, Kossol S, Kammer TH et al 1994. Proinflammatory cytokines in serum of patients with acute cerebral ischaemia: kinetics of secretion and relation to the extent of brain damage and outcome of disease. *J Neurol Sci* 122: 135-139.

Peeling RW, Wang SP, Grayston JT, Blasi F, Boman J, Clad A, Freidank H, Gaydos CA, Gnarp J, Hagiwara T, Jones RB, Orfila J, Persson K, Puolakkainen M, Saikku P, Schachter J 2000. *Chlamydia pneumoniae* serology: interlaboratory variation in microimmunofluorescence assay results. *Journal of Infectious Diseases* 181(suppl 3): S426-429.



Peltonen M, Rosen M, Lundberg V, Asplund K 2000. Social patterning of myocardial infarction and stroke in Sweden: incidence and survival. *Am J Epidemiol* 151:283-292.

Persson K, Boman J 2000. Comparison of five serologic tests for diagnosis of acute infections by *Chlamydia pneumoniae*. *Clinical and Diagnostic Laboratory Immunology* 7: 739-44.

Peter O, Dupuis G, Bee D, Luthy R, Nicolet J, Burgdorfer W 1988. Enzyme-linked immunosorbent assay for diagnosis of chronic Q fever. *J Clin Microbiol* 26:1978-1982.

Peter O, Dupuis G, Peacock MG, Burgdorfer W 1987. Comparison of enzyme-linked immunosorbent assay and complement fixation and indirect fluorescent-antibody tests for detection of *Coxiella burnetii* antibody. *J Clin Microbiol* 25:1063-1067.

Pickering T. Cardiovascular pathways: socioeconomic status and stress effects on hypertension and cardiovascular function. *Ann N Y Acad Sci* 896:262-277.

Piechowski-Jozwiak B, Mickielewicz A, Gaciong Z, Berent H, Kwiecinski H 2007. Elevated levels of anti-*Chlamydia pneumoniae* IgA and IgG antibodies in young adults with ischemic stroke. *Acta Neurologica Scandinavica* 116:144-149.

Pietroiusti A, Diomedi M, Silvestrini M et al 2002. Cytotoxin-associated gene-A-positive *Helicobacter pylori* strains are associated with atherosclerotic stroke. *Circulation* 106:580-584.

Pilote L, Green L, Joseph L, Richard H, Eisenberg MJ 2002. Antibiotics against *Chlamydia pneumoniae* and prognosis after myocardial infarction. *Am Heart J* 143:294-300.

Pinto AN 1996. AIDS and cerebrovascular disease. *Stroke* 27(3): 538-543.

Pislaru SV, Ranst MV, Pislaru C, Szelid Z, Theilmeier G, Ossewaarde JM, Holvoet P, Janssens S, Verbeken E, Van de Werf FJ 2003. *Chlamydia pneumoniae* induces neointima formation in coronary arteries of normal pigs. Cardiovascular Research 57:834-842.

Pönkä A, Jalanko H, Pönkä T, Stenvik M 1981. Viral and mycoplasmal antibodies in patients with myocardial infarction. Ann Clin Res 13:429-432.

Ponzetto A, Marchet A, Pellicano R et al 2002. Association of *Helicobacter pylori* infection with ischemic stroke of non-cardiac origin: the BATMAN project study. Hepatogastroenterology 49:631-634.

Pop GAM, Koudstaal PJ, Meeder HJ, Algra A, van Latum JC, van Gijn J, for the Dutch TIA Trial Study Group 1994. Predictive value of clinical history and electrocardiogram in patients with transient ischemic attack or minor ischemic stroke for subsequent cardiac and cerebral ischemic events. Arch Neurol 51:333-341.

Poshni IA, Millian SJ 1985. Seroepidemiology of *Legionella pneumophila* serogroup 1 in healthy residents of New York City. NY State J Med 85:10-14.

Poungvarin N 1998. Stroke in the developing world. Lancet 352: (suppl III) 19-22.

Prasad A, Zhu J, Halcox JPJ, Wacławski MA, Epstein SE, Quyyumi AA 2002. Predisposition to atherosclerosis by infections: role of endothelial dysfunction. Circulation 106:184-190.

Preusch MR, Grau AJ, Bugge F et al 2004. Association between cerebral ischemia and cytotoxin-associated gene-A-bearing strains of *Helicobacter pylori*. Stroke 35:1800-1804

Pugh PJ, Malkin CJ, Channer KS 2003. Cytomegalovirus seropositivity, infectious burden, and coronary artery disease. Am Heart J 145(2):e11.

Pussinen PJ, Alfthan G, Rissanen H, Reunanen A, Asikainen S, Knekt P 2004. Antibodies to periodontal pathogens and stroke risk. *Stroke* 35:2020-2023.

Rao SV, Schulman KA, Curtis LH, Gersh BJ, Jollis JG 2004. Socioeconomic status and outcome following acute myocardial infarction in elderly patients. *Archives of Internal Medicine* 164:1128-1133.

Rassu M, Cazzavillan S, Scagnelli M, Peron A, Bevilacqua PA, Facco M, Bertoloni G, Lauro FM, Zambello R, Bonoldi E 2001. Demonstration of *Chlamydia pneumoniae* in atherosclerotic arteries from various vascular regions. *Atherosclerosis* 158:73-79.

Rastawicki W, Kaluzewski S, Jagielski M 1998. Occurrence of serologically verified *Mycoplasma pneumoniae* infections in Poland in 1970-1995. *Eur J Epidemiol* 14:37-40.

Reik L 1993. Stroke due to Lyme disease. *Neurology* 43: 2705-2707.

Reinstein L, Gracey JG, Kline JA, et al 1972. Cardiac monitoring in the acute stroke patient. *Arch Phys Med Rehabil* 53 :311-314.

Reunanen A, Roivainen M, Kleemola M, Saikku P, Leinonen M, Hovi T, Knekt P, Leino A, Aromaa A 2002. Enterovirus, mycoplasma and other infections as predictors for myocardial infarction. *J Intern Med* 252:421-429.

Ridker PM, Hennekens CH, Buring JE, Kundsinn R, Shih J 1999. Baseline IgG antibody titers to *Chlamydia pneumoniae*, *Helicobacter pylori*, herpes simplex virus, and *cytomegalovirus* and the risk for cardiovascular disease in women. *Ann Intern Med* 131:573-577.

Ridker PM, Hennekens CH, Stampfer MJ, et al 1998. Prospective study of herpes simplex virus, cytomegalovirus, and the risk of future myocardial infarction and stroke. *Circulation* 98:2796-2799.

Rivers RPA, Hathaway WE, Weston WL 1975. The endotoxin-induced coagulant activity of human monocytes. *Br J Haematol* 30: 311-316.

Rojas F 2007. Poverty determinants of acute respiratory infections among Mapuche indigenous peoples in Chile's Ninth Region of Araucania, using GIS and spatial statistics to identify health disparities. *Int J Health Geogr* 6:26.

Rokitansky CV 1952. A manual of pathologic anatomy. London: The Sydenham Society, pp 265-275.

Romano F, Ribera G, D'Errico MM, Grasso GM, Montanaro D 1989. Levels of anti-Legionella antibodies in a healthy Neapolitan population (in Italian). *Annali d'igiene* 1:629-636.

Romeri E, Baker A, Griffiths C 2006. Mortality by deprivation and cause of death in England and Wales, 1999-2003. *Health Stat Q* 32:19-34.

Rosenfeld ME, Tsukada T, Gown AM, Ross R 1987. Fatty streak initiation in Watanabe heritable hyperlipaemic and comparably hypercholesterolaemic fat-fed rabbits *Arteriosclerosis* 7:9-23.

Ross R, 1993. The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature* 362:801-809.

Ross R, Glomset J 1973. Atherosclerosis and the arterial smooth muscle cell. Proliferation of smooth muscle is a key event in the genesis of the lesions of atherosclerosis. *Science* 180:1332-1339.

Rothenbacher D, Brenner H, Hoffmeister A, Mertens T, Persson K, Koenig W 2003. Relationship between infectious burden, systemic inflammatory response, and risk of stable coronary artery disease: role of confounding and reference group. *Atherosclerosis* 170:339-345.

Rothstein NM, Quinn TC, Madico G et al 2001. Effect of azithromycin on murine arteriosclerosis exacerbated by *Chlamydia pneumoniae*. J Infect Dis 183:232-238.

Rudd AG, Irwin, Rutledge Z et al 1999. The national sentinel audit for stroke: a tool for raising standards of care. J R Coll Physicians Lond 33: 460-464.

Ruiz-Beltran R, Herrero JI, Martin-Sanchez AM, Martin-Gonzalez JA 1990. Prevalence of antibodies to *Rickettsia conorii*, *Coxiella burnetii*, and *Rickettsia typhi* in Salamanca province (Spain). Serosurvey in the human population. Eur J Epidemiol 6:293-299.

Rupprecht HJ, Blankenberg S, Bickel C, Rippin G, Hafner G, Prellwitz W, Schlumberger W, Meyer J, for the AtheroGene Investigators 2001. Impact of viral and bacterial infectious burden on long-term prognosis in patients with coronary artery disease. Circulation 104:25-31.

Sacco RL, Ellenberg JH, Mohr JP et al 1989. Infarcts of undetermined cause: the NINCDS Stroke Data Bank. Ann Neurol 25:382-290.

Sako T, Takahashi T, Takehana K, Uchida E, Nakade T, Umemura T, Taniyama H 2002. Chlamydial infection in canine atherosclerotic lesions. Atherosclerosis 162:253-259.

Sander D, Winbeck K, Klingelhofer J et al 2002. Reduced progression of early carotid atherosclerosis after antibiotic treatment and *C pneumoniae* seropositivity. Circulation 106:2428-2433.

Sander D, Winbeck K, Klingelhofer J, Etgen T, Conrad B 2004. Progression of early carotid atherosclerosis is only temporarily reduced after antibiotic treatment of *Chlamydia pneumoniae* seropositivity. Circulation 109:1010-1015.

Sanzo JM, Garcia-Calabuig MA, Audicana A, Dehesa V 1993. Q fever: prevalence of antibodies to *Coxiella burnetii* in the Basque country. Int J Epidemiol 22:1183-1188.

Sasu S, LaVerda D, Qureshi N et al 2001. *Chlamydia pneumoniae* and chlamydial heat shock protein 60 stimulate proliferation of human vascular smooth muscle cells via toll-like receptor 4 and p44/p42 mitogen-activated protein kinase activation. *Circulation Research* 89:244-50.

Sawayama Y, Ariyama I, Hamada M, Otaguro S, Machi T, Taira Y, Hayashi J 2005. Association between chronic *Helicobacter pylori* infection and acute ischemic stroke: Fukuoka Harasanshin Atherosclerosis Trial (FHAT). *Atherosclerosis* 178:303-309.

Schiele F, Batur MK, Seronde MF, Meneveau N, Sewoke P, Bassignot A, Couetdic G, Caulfield F, Bassand JP 2001. Cytomegalovirus, *Chlamydia pneumoniae*, and *Helicobacter pylori* IgG antibodies and restenosis after stent implantation: an angiographic and intravascular ultrasound study. *Heart* 85:304-311.

Schleef RR, Bevilacqua MP, Sawdey M et al 1988. Cytokine activation of vascular endothelium. *J Biol Chem* 263: 5797-5803.

Schumacher A, Lerkerod AB, Seljeflot I, Sommervoll L, Holme I, Otterstad JE, Arnesen H 2001. *Chlamydia pneumoniae* serology: importance of methodology in patients with coronary heart disease and healthy individuals. *J Clin Microbiol* 39:1859-1864.

Selby G, Walker GL 1979. Cerebral arteritis in cat-scratch disease. *Neurology* 29: 1413-1418.

Sen S, Oppenheimer SM 1998. Cardiac disorders and stroke. *Current Opinion in Neurology* 11:51-56.

Shibutani T, Johnson TM, Yu ZX, Ferrans VJ, Moss J, Epstein SE 1997. Pertussis toxin-sensitive G proteins as mediators of the signal transduction pathways activated by cytomegalovirus infection of smooth muscle cells. *J Clin Invest* 100:2054-2061.

Shishehbor MH, Litaker D, Pothier CE, Lauer MS 2006. Association of socioeconomic status with functional capacity, heart rate recovery, and all-cause mortality. JAMA 295:784-792.

Shor A, Phillips J 2000. Histological and ultrastructural findings suggesting an initiating role for *Chlamydia pneumoniae* in the pathogenesis of atherosclerosis: a study of fifty cases. Cardiovas J S Afr 11:16-23.

Siegel S, Castellan NJ 1988. Nonparametric statistics for the behavioural sciences. New York: McGraw-Hill International Editions.

Sigal LH 1987. The neurologic presentation of vasculitic and rheumatologic syndromes. Medicine 66: 157-180.

Silver JG, Martin AW, McBride BC 1977. Experimental transient bacteremias in human subjects with varying degrees of plaque accumulation and gingival inflammation. J Clin Periodontol 4:92-99.

Sindre H, Haraldsen G, Beck S et al 2000. Human intestinal endothelium shows high susceptibility to cytomegalovirus and altered expression of adhesion molecules after infection. Scand J Immunol 51:354-360.

Sinisalo J, Mattila K, Valtonen V et al 2002. Effect of 3 months of antimicrobial treatment with clarithromycin in acute non-Q-wave coronary syndrome. Circulation 105:1555-1560

Sirmatel F, Muftuoglu MN, Tahtaci N, Abdurrahman N, Sirmatel O, Bulbul B 2003. *Chlamydia pneumoniae* seropositivity in patients with cerebral ischaemic attack with or without silent brain infarcts. Upsala J Med Sci 108:205-212.

Smeeth L, Thomas SL, Hall AJ, Hubbard R, Farrington P, Vallance P 2004. Risk of myocardial infarction and stroke after acute infection or vaccination. N Engl J Med 351:2611-2618.

Smieja M, Gnarpe J, Lonn E, Gnarpe H, Olsson G, Yi Q, Dzavik V, McQueen M, Yusuf S, for the Heart Outcomes Prevention Evaluation (HOPE) Study Investigators 2003. Multiple infections and subsequent cardiovascular events in the Heart Outcomes Prevention Evaluation (HOPE) Study. *Circulation* 107:251-257.

Smith EB 1990. Transport, interactions and retention of plasma proteins in the intima: the barrier function of the internal elastic lamina. *Eur Heart J Suppl* E:72-81.

Sorlie PD, Nieto FJ, Adam E, Folsom AR, Shahar E, Massing M 2000. A prospective study of cytomegalovirus, herpes simplex virus 1, and coronary heart disease: the atherosclerosis risk in communities (ARIC) study. *Arch Intern Med* 160:2027-2032.

Sotgiu S, Pugliatti, Rosati G, Deiana GA, Sechi GP 2003. Neurological disorders associated with *Mycoplasma pneumoniae* infection. *European Journal of Neurology* 10:165-168.

Speir E, Modali R, Huang ES, et al 1994. Potential role of human *cytomegalovirus* and p53 interaction in coronary restenosis. *Science* 265:391-394.

Speir E, Shibutani T, Yu ZX, Ferrans V, Epstein SE 1996. Role of reactive oxygen intermediates in cytomegalovirus gene expression and in the response of human smooth muscle cells to viral infection. *Circ Res* 79:1143-1152.

Speir E, Yu ZX, Ferrans VJ, Huang ES, Epstein SE 1998. Aspirin attenuates cytomegalovirus infectivity and gene expression mediated by cyclooxygenase-2 in coronary artery smooth muscle cells. *Circ Res* 83:210-216.

Spodick DH, Flessas AP, Johnson MM 1984. Association of acute respiratory symptoms with onset of acute myocardial infarction: prospective investigation of 150 consecutive patients and matched control patients. *Am J Cardiol* 53:481-482.

Stanger OH, Semmelrock HJ, Rehak P, Tiran B, Meinitzer A, Rigler B, Tiran A 2002. Hyperhomocyst(e)inemia and *Chlamydia pneumoniae* IgG seropositivity in patients with coronary artery disease. *Atherosclerosis* 162:157-162.



Sary HC, Chandler B, Dinsmore RE et al 1995. A definition of advanced types of atherosclerotic lesions and a histological classification of atherosclerosis. *Atherosclerosis Thromb Vasc Biol* 15:1512-1531.

Sary HC, Chandler B, Glakov S et al 1994. A definition of initial, fatty streak, and intermediate lesions of atherosclerosis. *Circulation* 89:2462-2478.

Steinberg D, Parthasarathy S, Carew TE et al 1989. Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity. *N Engl J Med* 320:915-924.

Stephoe A, Shamaei-Tousi A, Gylfe A, Henderson B, Bergstrom S, Marmot M 2007. Socioeconomic status, pathogen burden and cardiovascular disease risk. *Heart* 93:1567-1570.

Stjarne MK, de Leon AP, Hallqvist J, for the SHEEP study group 2004. Contextual effects of social fragmentation and material deprivation on risk of myocardial infarction- results from the Stockholm Heart Epidemiology Program (SHEEP). *Int J Epidemiol* 33:732-741.

Stout JE, Yu VL 1997. Legionellosis. *N Engl J Med* 337:682-687.

Stout RW 1998. Atherosclerosis and lipid metabolism; in Tallis RC, Fillit HM, Brocklehurst JC (eds): *Brocklehurst's Textbook of Geriatric Medicine and Gerontology*. Churchill Livingstone, 5<sup>th</sup> Edition, pp 263-279.

Suarez-Estrada, Rodriguez-Barbosa JI, Gutierrez-Martin CB, Castaneda-Lopez MR, Fernandez-Marcos JM, Gonzalez-Llamazares OR, Rodriguez-Ferri EF 1996. Seroepidemiological survey of Q fever in Leon province, Spain. *Eur J Epidemiol* 12:245-250.

Svanbom M 1980. A prospective study on septicaemia. II. Clinical manifestations and complications, results of antimicrobial treatment and report of a follow-up study. *Scan J Infect Dis* 12: 189-206.

Syrjanen J 1989. Central nervous system complications in patients with bacteraemia. *Scan J Infect Dis* 21: 285-296.

Syrjanen J 1989. Central nervous system complications in patients with bacteraemia. *Scan J Infect Dis* 21: 285-296.

Syrjanen J, Valtonen VV, Iivanainen M, Kaste M, Huttunen JK 1988. Preceding infection as an important risk factor for ischaemic brain infarction in young and middle aged patients. *BMJ* 296:1156-1160.

Szczudlik A, Slowik A, Turaj W, Zwolinska G, Wyrwicz-Petkow U, Kasprzyk K, Bosak M 2000. Early predictors of 30-day mortality in supratentorial ischaemic stroke patients – first episode. *Med Sci Monit* 6:75-80.

Tanir G, Aydemir C, Yilmaz D, Tuygun N 2006. Internal carotid artery occlusion associated with *Mycoplasma pneumoniae* infection in a child. *Turk J Pediatr* 48:166-171.

Tanne D, Haim M, Boyko V et al 2003. Prospective study of *Chlamydia pneumoniae* IgG and IgA seropositivity and risk of incident ischemic stroke. *Cerebrovasc Dis* 16:166-170.

Tatschl C, Stollberger C, Matz K, Yilmaz N, Eckhardt R, Nowotny M, Dachenhausen A, Brainin M 2006. *Cerebrovasc Dis* 21:47-53.

Tavendale R, Parratt D, Brook RA, Tunstall-Pedoe H 1999. Antibodies to *Chlamydia pneumoniae* do not predict subsequent CHD in the Scottish Heart Health and MONICA studies [Abstract No P2221]. *Eur Heart J* 20(suppl):450.

Taylor RG, Lewis JC 1986. Endothelial cell proliferation and monocyte adhesion to atherosclerotic lesions of white carneau pigeons. *Am J Pathol* 125:152-160.

Taylor-Robinson D, Thomas BJ 1998. *Chlamydia pneumoniae* in arteries: the facts, their interpretation, and future studies. *J Clin Pathol* 51:793-797.

Taylor-Robinson D 1996. Infections due to species of *Mycoplasma* and *Ureaplasma*: an update. *Clin Infect Dis* 23:671-684.

Tegos TJ, Kalodiki E, Sabetai MM, Nicolaides AN 2001. The genesis of atherosclerosis and risk factors: a review. *Angiology* 52:89-98.

The English Indices of Deprivation 2004. London, UK: Neighbourhood Renewal Unit, Office of the Deputy Prime Minister. Available at: <http://www.communities.gov.uk/archived/general-content/communities/indicesofdeprivation/216309/>

Thomas DR, Treweek L, Salmon RL, Kench SM, Coleman TJ, Meadows D, Morgan-Capner P, Caul EO 1995. The risk of acquiring Q fever on farms: a seroepidemiological study. *Occup Environ Med* 52:644-647.

Tissot-Dupont H, Raoult D, Brouqui P, Janbon F, Peyramond D, Weiller PJ, Chicheportiche C, Nezri M, Poirier R 1992. Epidemiologic features and clinical presentation of acute Q fever in hospitalised patients: 323 French cases. *Am J Med* 93:427-434.

Tissot-Dupont H, Torres S, Nezri M, Raoult D 1999. Hyperendemic focus of Q fever related to sheep and wind 1999. *Am J Epidemiol* 150:67-74.

Tompkins LS, Schachter J, Boman J, Dowell S, Gaydos CA, Levison ME, Maass M, Madico G, Orfila J, Ouchi K, Peeling RW, Taylor-Robinson D, Stamm WE, Wang SP, Blasi F, Relman D 2000. Collaborative multidisciplinary workshop report: detection, culture, serology, and antimicrobial susceptibility testing of *Chlamydia pneumoniae*. *J Infect Dis* 181:S460-461.

Townsend P, Phillimore P, Beattie A 1987. Health and deprivation: inequality and the north. London: Croome Helm.

Tsiodras S, Kelesidis I, Kelesidis T, Stamboulis E, Giamarellou H 2005. Central nervous system manifestations of *Mycoplasma pneumoniae* infections. Journal of Infection 51 :343-354.

Tuuminen T, Palomaki P, Paavonen J 2000(a). The use of serologic tests for the diagnosis of chlamydial infections. Journal of Microbiological Methods 42:265-79.

Tuuminen T, Varjo S, Ingman H, Weber T, Oksi J, Viljanen M 2000(b). Prevalence of *Chlamydia pneumoniae* and *Mycoplasma pneumoniae* immunoglobulin G and A antibodies in a healthy Finnish population as analyzed by quantitative enzyme immunoassays. Clin Diagn Lab Immunol 7:734-738.

US Department of Labor, 2000. Standard Occupational Classification (SOC) System. <http://www.bls.gov/soc/home.htm>

Vainas T, Stassen FRM, Schurink GWH, Tordoir JHM, Welten RJThJ, van den Akker LHJM, Kurves HAJM, Bruggeman CA, Kitslaar PJEHM 2005. Secondary prevention of atherosclerosis through *Chlamydia pneumoniae* eradication (SPACE trial): a randomised clinical trial in patients with peripheral arterial disease. European Journal of Vascular and Endovascular Surgery 29:403-411.

Valente AJ, Rozek MM, Sprague EA et al 1992. Mechanisms in intimal monocyte-macrophage recruitment. Circulation 86(suppl III):20-25

Vallance P, Collier J, Bhagat K 1997. Infection, inflammation, and infarction: does acute endothelial dysfunction provide a link? Lancet 349:1391-1392.

Valtonen V, Kuikka A, Syrjanen J 1993. Thrombo-embolic complications in bacteraemic infections. European Heart Journal 14(Supplement K): 20-23.

Vammen S, Lindholt JS, Ostergaard L et al 2001. Randomised double-blind controlled trial of roxithromycin for prevention of abdominal aortic aneurysm expansion. *British Journal of Surgery* 88:1066-72.

Van Dam-Mieras MC, Muller AD, van Hinsbergh VW et al 1992. The procoagulant response of cytomegalovirus infected endothelial cells. *Thromb Haemost* 68:364-370.

Van der Poll T, Buller HR, ten Gate H et al 1990. Activation of coagulation after administration of tumour necrosis factor to normal subjects. *N Engl J Med* 322: 1622-1627.

van Rossum CTM, van de Mheen H, Breteler MMB, Grobbee DE, Mackenbach JP 1999. Socioeconomic differences in stroke among Dutch elderly women: the Rotterdam study. *Stroke* 30:357-362.

Vasthare US, Heinel LA, Rosenwasser RH, Turns RF 1990. Leucocyte involvement in cerebral ischaemia and reperfusion injury. *Surg Neurol* 33: 261-265.

Veldhuyzen van Zanten SJ, Sherman PM 1994. *Helicobacter pylori* infection as a cause of gastritis, duodenal ulcer, gastric cancer and nonulcer dyspepsia: a systematic overview. *CMAJ* 150:177-185.

Ventura HO, Mehra MR, Smart FW, Stapleton DD 1995. Cardiac allograft vasculopathy: current concepts. *Am Heart J* 129:791-798.

Verkooyen RP, Willemse D, Hiep-van Casteren SCAM, Mousavi Joulandan SA, Snijder RJ, van den Bosch JMM, van Helden HPT, Peeters MF, Verbrugh HA 1998. Evaluation of PCR, culture, and serology for diagnosis of *Chlamydia pneumoniae*. *Journal of Clinical Microbiology* 36: 2301-7.

Vernino S, Brown RD Jr, Sejvar JJ, Sicks JD, Petty GW, O'Fallon WM 2003. Cause-specific mortality after first cerebral infarction: a population-based study. *Stroke* 34:1828-1832.

Vink A, Poppen M, Schoneveld AH et al 2001. Distribution of *Chlamydia pneumoniae* in the human arterial system and its relation to the local amount of atherosclerosis within the individual. *Circulation* 103:1613-7.

Virok D, Kis Z, Karai L et al 2001. *Chlamydia pneumoniae* in atherosclerotic middle cerebral artery. *Stroke* 32:1973-1976.

Visudhiphan P, Chiemchanya S, Sirinavin S 1992. Internal carotid artery occlusion associated with *Mycoplasma pneumoniae* infection. *Pediatr Neurol* 8:237-239.

Von Reyn CF, Levy BS, Arbeit RD et al 1981. Infective endocarditis: an analysis based on strict case definitions. *Ann Intern Med* 94: 505-518.

Waag D, Chulay J, Marrie T, England M, Williams J 1995. Validation of an enzyme immunoassay for serodiagnosis of acute Q fever. *Eur J Clin Microbiol Infect Dis* 14:421-427.

Wade DT 1994. Stroke (acute cerebrovascular disease). In: Stevens A, Raftery J (eds). *Health Care Needs Assessment*. Oxford: Radcliffe Medical Press.

Waitzman N, Smith K 1998. Phantom of the area: poverty-area residence and mortality in the United States. *Am J Public Health* 88:973-976.

Wang JH, Liu YC, Cheng DL, Yeng MY, Chen YS, Chen BC 1993. Seroprevalence of *Chlamydia pneumoniae* in Taiwan. *Scan J Infect Dis* 25:565-568.

Warlow CP 1998. Stroke: epidemiology of stroke. *Lancet* 352(suppl III):1-4.

Warlow CP, Dennis MS, van Gijn J et al 1996. A practical approach to the management of stroke patients. In: *Stroke: A practical guide to management*. Oxford: Blackwell Science; 192, 271-272, 422-423.

Watkins-Riedel T, Stanek G, Daxboeck F 2001. Comparison of SeroMP IgA with four other commercial assays for serodiagnosis of *Mycoplasma pneumoniae* pneumonia. *Diagn Microbiol Infect Dis* 40:21-25.

Weir NU, Gunkel A, McDowall M, Dennis MS 2005. Study of the relationship between social deprivation and outcome after stroke. *Stroke* 36:815-819.

Wells BJ, Mainous III AG, Dickerson LM 2004. Antibiotics for the secondary prevention of ischemic heart disease: a meta-analysis of randomized controlled trials. *Archives of Internal Medicine* 164:2156-2161.

West R 2000. Commentary: adjustment for potential confounders may have been taken too far. *BMJ* 321:213.

Whincup PH, Mendall MA, Perry IJ, Strachan DP, Walker M 1996. Prospective relations between *Helicobacter pylori* infection, coronary heart disease, and stroke in middle aged men. *Heart* 75:568-572.

Wiesli P, Czerwenka W, Meniconi A, Maly FE, Hoffmann U, Vetter W, Schulthess G 2002. Roxithromycin treatment prevents progression of peripheral arterial occlusive disease in *Chlamydia pneumoniae* seropositive men: a randomised, double-blinded, placebo-controlled trial. *Circulation* 105:2646-2652.

Williams RC 1990. Periodontal disease. *N Engl J Med* 322:373-382.

Wilson SH, Caplice NM, Simari RD, Holmes DR Jr, Carlson PJ, Lerman A 2000. Activated nuclear factor  $\kappa$ B is present in the coronary vasculature of experimental hypercholesterolemia. *Atherosclerosis* 148:23-30.

Wimmer MLJ, Sandmann-Strupp R, Saikku P, Haberl RL 1996. Association of chlamydial infection with cerebrovascular disease. *Stroke* 27:2207-2210.

Wolf PA 1998. Stroke: prevention of stroke. *Lancet* 352(suppl III):15-18.

Wong KYK, Mac Walter RS, Douglas D, Fraser HW, Ogston SA, Struthers AD 2003. Long QT<sub>c</sub> predicts future cardiac death in stroke survivors. *Heart* 89:377-381.

Wong KYK, Wong SYS, Fraser HW, Ersoy Y, Ogston S, Wolfson D, Malek M, Struthers AD, Tunstall-Pedoe H, MacWalter RS 2006. Effect of social deprivation on mortality and the duration of hospital stay after a stroke. *Cerebrovasc Dis* 22:251-257.

Woodhouse PR, Khaw KT, Plummer M, Foley A, Meade TW 1994. Seasonal variation of plasma fibrinogen and factor VII activity in the elderly: winter infections and death from cardiovascular disease. *Lancet* 343:435-439.

Woodward M 1992. Formulae for sample size, power and minimum detectable relative risk in medical studies. *The Statistician* 41:185-196.

Woolf N 1990. Pathology of atherosclerosis. *Br Med Bull* 46:960-985.

World Health Organisation 1978. Cerebrovascular disease: a clinical and research classification. WHO Offset Publ 43.

Wu T, Trevisan M, Genco RJ, Dorn JP, Falkner KL, Sempos CT 2000. Periodontal disease and risk of cerebrovascular disease: the first National Health and Nutrition Examination Survey and its follow-up study. *Arch Intern Med* 160:2749-2755.

Yonemitsu Y, Kaneda Y, Komori K, Hirai K, Sugimachi K, Sueishi K 1997. The immediate early gene of human cytomegalovirus stimulates vascular smooth muscle cell proliferation in vitro and in vivo. *Biochem Biophys Res Commun* 231:447-451.

Yonke CA, Stiefel HE, Wentworth BB, Wilson DL 1982. Prevalence of antibody to serogroups 1-4 of *Legionella pneumophila*. A seroepidemiologic study using the indirect hemagglutination test. *Am J Epidemiol* 115:633-639.

Yucesan C, Sriram S 2001. *Chlamydia pneumoniae* infection of the central nervous system. *Curr Opin Neurol* 14:355-359.



Zahn R, Schneider S, Frilling B et al 2003. Antibiotic therapy after acute myocardial infarction: a prospective randomised study. *Circulation* 107:1253-1259.

Zhang Y, Cliff WJ, Schoefl GI et al 1993. Plasma protein insudation as an index of early coronary atherogenesis. *Am J Pathol* 143:496-506.

Zhang ZH, Rashba S, Oppenheimer SM 1998. Insular cortex lesions after baroreceptor sensitivity in the urethane-anesthetised rat. *Brain Res* 813:73-81.

Zhou G, Liu X, Xu G, Liu X, Zhang R, Zhu W 2006. The effect of socioeconomic status on three-yaer mortality after first-ever ischaemic stroke in Nanjing China. *BMC Public Health* 6:227.

Zhu J, Nieto FJ, Horne BD, Anderson JL, Muhlestein JB, Epstein SE 2001. Prospective study of pathogen burden and risk of myocardial infarction or death. *Circulation* 103:45-51.

Zhu J, Quyyumi AA, Norman JE, Csako G, Waclawiw MA, Shearer GM, Epstein SE 2000. Effects of total pathogen burden on coronary artery disease risk and C-reactive protein levels. *Am J Cardiol* 85:140-146.